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Rapid Identification of Shigella in a Public Health Laboratory. W. W. Ferguson, Ph.D., Mary Branston, A.B., Grace L. McCallum, B.S., and Margaret J. Carlson, B.S., Lansing, Mich. ---- 349

The Serologic Response in Murine Typhus as Measured by the Weil-Felix, Rickettsial Complement Fixation, and Rickettsial Agglutination Reactions. Carl T. Nelson, M.D., New York, N. Y. ---- 360

Influenza B in Needham, Massachusetts, December, 1945. Maxwell Finland, Mildred W. Barnes, Manson Meads, Edwin M. Ory, and A. Daniel Rubenstein, Boston, Mass. ---- 367

The Effect of Antihistamine Drugs Upon Serum-Induced Myocarditis in Rabbits. Franklin A. Kyser, M.D., John C. McCarter, M.D., and James Stengle, M.D., Evanston, Ill. ---- 379

Evidence for the Activity of a Second Member of the Vitamin M Group (Fermentation Factor) in Sprue. Edgar Jones, M.D., Henry F. Warden, M.D., and William J. Darby, M.D., Ph.D., Nashville, Tenn. ---- 387

Thiouracil and Propylthiouracil: A Comparative Clinical Study. William H. Beierwaltes, M.D., and Cyrus C. Sturgis, M.D., Ann Arbor, Mich. ---- 392

Observations on the Diurnal Excretion of Urobilinogen in the Urine of Normal Subjects and of Patients With Laennec's Cirrhosis. Edmund Pellegrino, Arthur J. Patek, Jr., Anne Colcher, and Beatrice Domanski, New York, N. Y. 397

A Comparison of the Nutritive Value of the Proteins in Mixed Diets for Dogs, Rats, and Human Beings. D. M. Hegsted, Virginia Kent, A. G. Tsongas, and F. J. Stare, Boston, Mass. ---- 403

Prothrombin Time Studies on Human Beings and Experimental Animals. G. A. Nitshe, Jr., H. W. Gerarde, and H. F. Deutsch, Madison, Wis. ---- 410

The Corpuscular Constants of College Women of the North Central States. Gladys Kinsman Lewis, Margaret A. Ohlson, Dena Cederquist, and Eva G. Donelson 419

Two Popular Fallacies Regarding Rh. First Lieutenant Paul G. Hattersley, Medical Corps, Army of the United States ---- 423

(Contents continued on inside front cover)

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Progress

Metabolic Complexities of Pellagra. Philip Handler, Ph.D., Durham, N. C. 428

Nitrogen Balance. Philip Handler, Ph.D., Durham, N. C. 437

Laboratory Methods

Assay of Penicillin, Streptomycin, Trivalent Organic Arsenicals, and Other Bactericidal and Bacteriostatic Agents. Edwin E. Osgood, M.D., Portland, Ore. With the Technical Assistance of Barbara Gamble, B.S. 444

An Experimental Method for Obtaining an Ultrafiltrate of the Blood. M. R. Malinow and W. Korzon, Chicago, Ill. 461

Book Reviews

Book Reviews 472

Announcement

Announcement 474

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RAPID IDENTIFICATION OF SHIGELLA IN A PUBLIC HEALTH LABORATORY

W. W. Ferguson, Ph.D., Mary Branston, A.B., Grace L. McCallum, B.S., and Margaret J. Carlson, B.S.
Lansing, Mich.

BOYD'S concept of the antigenic structure of Flexner dysentery bacteria has been given practical application in this country by Wheeler.[1] He[2] recognized that the essential differences between Flexner types of Shigella are the specific antigens, while the group components are of secondary importance, except perhaps in the differentiation of Flexner W into subtypes. Wheeler[1] has described methods for serologic identification of other pathogenic Shigella, as well as the Flexner and Boyd types,* by means of a slide agglutination test employing formalinized antigens and properly absorbed sera. He has specified, however, that "classification of the organisms as determined by the agglutination test should be confirmed by fermentation reactions."

Weil and associates[3] are in apparent agreement with Boyd and Wheeler that specific or "primary" antigens distinguish the Flexner as well as the Boyd types from each other. However, some of the dual antigens recognized by Weil and co-workers would be regarded, necessarily, as group antigens by Wheeler. In effect, the former authors have combined the concept of Boyd with that of Andrewes and Inman.[4]

Weil and associates[3] have advocated the use of absorbed sera for rapid identification of Shigella. More recently, Weil and co-workers[7] have established the existence of minor antigens which interrelate *Shigella ambigua, Shigella alkalescens,* and *Shigella dispar* with certain of the Boyd types. They caution that species (and type) identification must rest on both immunologic and cultural properties.

Nelson and associates,[8] who examined 450 "presumptively positive" enteric cultures in India, feel that the use of absorbed sera in the spot agglutination test for identification of Shigella is far superior to the use of unabsorbed sera in the conventional tube agglutination method. Eighty per cent of the cultures examined by these investigators were identified as Shigella, presumably by serologic means alone.

Extensive use of Shigella typing sera was made in Army laboratories in various parts of the world during World War II. Outside of one brief publication,[9] no information on the sera and their uses has appeared in the literature.

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*Throughout this paper the Flexner types will be designated as Flexner V, W, X, Y, and Z and the Boyd types as 103, P119, 88, 170, P288, D1, D19, P274, and P143. At present the different systems for designating Flexner and Boyd types, as proposed by Boyd,[5] Wheeler,[2] and Weil and associates,[3] are in disagreement. The effect on the literature of the various systems may be judged from the following example. Francis[6] has announced the discovery of two new antigenic types of Shigella with the provisional designations of *Shigella flexneri* type VII and *Sh. flexneri* type VIII. According to Weil types VII and VIII are the same as the Flexners X and Y of Andrewes and Inman[4] which Weil and associates regard as types possessing specific antigens.

The Army sera were made for use in a slide agglutination technique for provisional identification of dysentery organisms, to be followed by determination of motility and confirmatory biochemical studies.

Our experience[10] in this laboratory in the preparation and use of Flexner and Boyd typing sera led us to believe that Wheeler's views on the serology of the Flexner group were better applicable to diagnostic problems than those of Weil and associates.[3] It appeared to us that Wheeler's absorption scheme could be extended and that antisera could be produced which would be free of practically all minor agglutinins. A Flexner antiserum, for example, could be absorbed not only with other cross-reacting heterologous Flexner types for removal of group agglutinins but with any heterologous Shigella which gave a reaction in the serum. Such an antiserum should be free of agglutinins for all dysentery organisms except the homologous type. Absorbed antiserum for other species, such as *Shigella dysenteriae,* could be produced by the same method. It was recognized that paracolon and other organisms of the Enterobacteriaceae might possess minor antigens in common with Shigella. Only trial of absorbed sera could determine if cross-reactions would occur with bacteria of other genera.

During a period of preliminary trial the typing of dysentery bacteria was attempted with absorbed sera only after biochemical studies had been made. When no disagreements between cultural and serologic tests were noted during the period of trial, it was decided to classify Shigella on the basis of serologic reactions alone.

This paper reports the examination of 315 enteric cultures by slide agglutination technique with absorbed Shigella sera. By means of this rapid method of examination a report was made on the identity of Shigella present in a fecal specimen approximately forty-eight hours after the specimen was received in the laboratory. Eventual fermentation and tube agglutination studies were made on every organism included in the study.

METHODS

Sera.—The following absorbed sera were provided the Central Diagnostic Laboratory: *Sh. dysenteriae, Sh. ambigua, Sh. alkalescens* (Type I), *Shigella sonnei,* polyflexner (prepared with V, W, X, Y, Z, 103, P119, and 88 strains), monovalent Flexner V, monovalent Flexner W, monovalent Flexner Z, monovalent Boyd 103, monovalent Boyd P119, and monovalent Boyd 88. Additional absorbed sera were prepared for the mannitol-negative types Sachs Q454, Q771, Q902, Q1030, and Q1167, together with absorbed sera for Boyd types 170, P288, D1, D19, P274, and P143. A polyvalent serum was also prepared to cover Boyd types 170 through P143. The Sachs and Boyd type sera, other than Boyd 103, P119 and 88, were not used routinely.

Source of Cultures.—The same stock strains used for production of sera in an earlier study[10] were employed, plus additional cultures.*

*Contributed by Dr. Kenneth M. Wheeler, Connecticut State Department of Health, Hartford; Captain C. V. Seastone, Medical Corps, Army Medical Center, Washington, D. C.; Dr. C. A. Peluffo and Dr. Estenio Hormaeche, Institute of Hygiene, Montevideo, Uruguay; and Dr. Joseph Felsen, The Bronx Hospital, New York City.

Production of Antisera.—White rabbits weighing approximately 6 pounds were used for production of antisera. The usual immunization procedure was as follows: A series of three intracutaneous inoculations were made on alternate days with formalinized saline suspensions of cultures grown on blood agar medium. These were followed by six to seven intravenous inoculations of living organisms also grown on blood agar. Intravenous inoculations were made on alternate days.

Suspensions were standardized to the turbidity of a No. 2 McFarland standard, and care was taken not to suspend agar along with organisms. The total amount of inoculum was usually 9.0 cubic centimeters.

Immune sera after processing were preserved with chloroform.

Preparation of Absorbed Sera.—Table I on the preparation of a Flexner V antiserum will serve as an example of the steps taken in preparing an absorbed serum. Unabsorbed serum, diluted 1:10, was tested by slide agglutination technique against eighteen-hour, living, smooth strains of Shigella grown on blood agar. None of the cultures was heated before use to destroy heat-labile "blocking" antigen, since the cultures listed, including *Sh. alkalescens* R strain, have shown no evidence of possessing such a substance.

The technique of the test was the same as that employed in identification of unknown dysentery cultures by the finished serum. The wells of agglutination slides* were filled two-thirds full of serum and test organisms were rubbed into the edges of serum drops by means of a loop or straight inoculating wire. An even, milky suspension of organisms resulted which was, admittedly, somewhat uneven in turbidity from well to well. The slide was rocked by hand and observed for fifteen minutes. The system of recording reactions was by plus signs: 4 plus denoted complete agglutination, 2 plus partial agglutination, doubtful a barely detectable clumping. Since speed of reaction was some indication of the potency of a serum, the words "instant," "moderate," and "slow" were used to denote reactions taking place, respectively, as soon as suspension was accomplished, within one minute, or after approximately five minutes.

Following the determination of reactions in unabsorbed serum, strains were selected for removal of cross-reacting agglutinins. With Flexner antisera the X and Y strains were always used for initial absorptions. Organisms for absorption were grown on veal infusion agar in Blake bottles; they were then washed off the agar with saline and spun down in an anglehead centrifuge. The supernatant fluid was poured off and unabsorbed serum, diluted 1:10, was added to the packed bacteria. Resuspension of the bacteria was accomplished by stirring. Incubation was carried out at 50° C. for one hour. An excess of the absorbing dose of bacteria was used for each step in freeing a serum of cross-agglutinins.

After incubation of serum and antigen, bacterial cells were removed by centrifugation, and the serum was tested against each strain which had reacted in the initial titration. In the case of Flexner V antiserum, Lot 3, absorption was completed by a second absorbing dose consisting of Flexner Z and *Sh. alkalescens* organisms. Slide agglutination tests were carried out with Flexner V antiserum and the antigens which had reacted following absorption with X

*Supplied by Clay-Adams Company, Inc., New York, N. Y.

TABLE I. FLEXNER V ANTISERUM, LOT 3

LIVING ANTIGENS	SLIDE AGGLUTINATION REACTIONS			
	UNABSORBED SERUM (DILUTION 1:10)	FOLLOWING FIRST ABSORPTION* (DILUTION 1:10)	FOLLOWING SECOND ABSORPTION† (DILUTION 1:10)	ABSORBED SERUM (DILUTION 1:20)
Flexner V, 3090	++++	++++ Instant	++++ Instant	++++ Moderate
Flexner V, R	++++	++++ Instant	++++ Instant	++++ Moderate
Flexner WIIa, R	++	-		
Flexner WIIb, C	++	-		
Flexner X694, C	++++	-		
Flexner X1533, C	++++	-		
Flexner X1806, C	++++	-		
Flexner X1807, C	++++	-		
Flexner Y412, A	++++	-		
Flexner Y, R	++++	-		
Flexner Y F1219	++++	-		
Flexner Z, R	++++	++++	-	
Boyd 103, C	++++	++++	-	
Boyd P119, M	++	-		
Boyd P119, A	++	-		
Boyd 88, C	++	-		
Boyd 170, C	++	-		
Boyd P288, M	-	-		
Boyd P288, N.M.	-	-		
Boyd D1, M	-	-		
Boyd D19, M	+	-		
Boyd P274, M	-	-		
Boyd P143, M	±	-		
Lavington 2525	-	-		
Sachs Q454	-	-		
Sachs Q771	-	-		
Sachs Q902	-	-		
Sachs Q1030	-	-		
Sachs Q1167	-	-		
Sh. dysenteriae 1046	-	-		
Sh. ambigua H	-	-		
Paracolon 339	++	-		
Sh. sonnei	-	-		
Sh. alkalescens R	++++	++++	-	
Salmonella typhi 703	-	-		

++++, Complete agglutination.
++, Agglutination, 50 per cent.
Titer of Flexnor V antiserum, absorbed, with homologous strains, 1-2560.
Determination made by tube dilution method, incubation 50° C. for eighteen hours.
*Absorbing strains: Flexners X694, X1533, X1806, X1807, Y R, Y F1219, and Y412.
†Absorbing strains: Flexner Z and *Sh. alkalescens*.

and Y strains. At this stage the serum appeared free of minor agglutinins, while the reaction with Flexner V organisms was instant and complete. The serum dilution was doubled and tests were again made with Flexner V antigens. When agglutination took place within one minute and was complete, a dilution of 1:20 was selected as the "working dilution."

Only rarely has it been necessary to accept an antiserum diluted 1:10; on the other hand, no serum was diluted more than 1:20 because of possible deterioration. As a preservative, merthiolate in powder form was added to antiserum to make a final concentration of 1:1,000. It was then filtered through a Seitz pad and a final titration made by testing the serum against its homologous strains by tube agglutination technique.

In the example given in Table I, a total of thirty-five strains are listed as antigens. The number has varied from lot to lot of antisera, as new antigenic types have been added to our collection. At present we employ forty-six strains as titration antigens. Storage of these strains dried in vacuo from the frozen state has aided greatly in prevention of dissociation.

The inclusion of *Salmonella typhi* among the stock strains was done originally as an experiment. It was thought a worth-while precaution to remove minor agglutinins for *S. typhi* from Shigella sera since identification of dysentery cultures was to be made from triple sugar slants and since occasional strains of the typhoid organism are found which are dysentery-like in their reactions, failing to produce H_2S in twenty-four hours. When it was found that strong cross-reactions for *S. typhi* develop in some of the Flexner sera, *S. typhi* 703 was included in all titrations. Whether or not the cross-reactions with *S. typhi* are due to antibodies similar to the alpha agglutinins reported by Stamp and Stone[11] has not been determined.

Performance of the Diagnostic Slide Agglutination Test.—The technique of the diagnostic slide agglutination test has varied somewhat with the bacteriologist. In general the method has been as follows: Organisms producing dysentery-like reactions on triple sugar slants were selected for typing. Each culture was tested in trypaflavine solution (1:500 concentration of acriflavine hydrochloride in 0.85 per cent NaCl solution) and in physiologic saline solution for indications of autoagglutinability. It was discovered early in our experience with trypaflavine solution that *Sh. alkalescens* strains possessing heat-labile "blocking" substance were clumped rapidly in trypaflavine,* yet remained in even suspension in normal saline. Organisms clumping in trypaflavine and not in saline were either (1) placed in saline suspension and boiled for fifteen minutes, then tested with absorbed *Sh. alkalescens* antiserum; or (2) identified by conventional methods, that is, by observation of biochemical activities and reaction in specific serum by tube method. Only strains of *Sh. alkalescens* identified by slide agglutination are included in this report.

Cultures which did not clump in either trypaflavine or saline were tested in polyflexner, *Sh. sonnei,* and *Sh. alkalescens* absorbed sera. Organisms which were agglutinated in either of the last two sera were reported by species. A culture which agglutinated in polyflexner serum was tested in the three monovalent Flexner (V, W, and Z) and three Boyd sera (103, P119, and 88). An organism which reacted in the polyvalent serum and a monovalent serum was reported by species and type, that is, *Sh. paradysenteriae,* Boyd 103. With but few exceptions the reactions in monovalent sera were complete in one or two minutes or less. Flexner W strains were more variable in agglutinability than other types. Occasional strains were clumped rapidly; others, while smooth by test in saline, were slow to react.

Biochemical Tests.—All cultures discussed in this report were tested for fermentative reactions in semisolid agar medium containing glucose, lactose, sucrose, maltose, mannitol, and xylose, respectively. Organisms selected for

*Since some *Sh. dysenteriae, Sh. ambigua,* and Sachs strains have been reported as occurring with heat-labile antigens, it is possible that occasional strains will clump in trypaflavine. VI strains of *S. typhi* clump rapidly in trypaflavine.[12-14]

special study, such as strains of Boyd 88 and *Sh. alkalescens,* were inoculated in liquid carbohydrate media. In the case of Boyd 88 cultures, dulcitol was added to the list of carbohydrates. An occasional strain of particular interest, such as C-27, together with strains of *Sh. alkalescens,* were tested with a wide range of carbohydrates. Observation of indol production and hydrolysis of urea was done routinely, whereas reactions on Simmons' citrate were observed only on cultures selected for special study. Tests for motility were made on *Sh. alkalescens* cultures and on all strains whose biochemical pattern was at variance with the evidence of tube or slide agglutination.

Macroscopic Agglutination.—Tube agglutination tests were carried out at 50° C. in the water bath for eighteen hours on all organisms with dysentery-like reactions in triple and single sugar media. The following antisera were available: *Sh. dysenteriae, Sh. ambigua, Sh. alkalescens, Sh. sonnei,* and polyflexner.

RESULTS

A total of 315 cultures which gave dysentery-like reactions in triple sugar agar slants was examined by slide agglutination technique with absorbed sera. Of these, 307 were accepted as Shigella on the basis of their agglutination in the spot test. The serologic identification of the 307 cultures was as follows:

Sh. sonnei	159
Sh. paradysenteriae (Flexner and Boyd)	115
Sh. alkalescens	32
Sh. ambigua	1
Total	307

The paradysentery types were identified by monovalent sera as:

Flexner V	1
Flexner W	26
Flexner Z	28
Boyd 103	8
Boyd P119	0
Boyd 88	52
Total	115

Five cultures which are included in the total of 315 were agglutinated in polyflexner antiserum on slide test. Four of these failed to react in monovalent sera and were, therefore, not reported as Shigella. Fermentation studies revealed that they were aerogenic and belonged to the paracolon group. The fifth culture, 8255, reacted slowly in Flexner W antiserum and was reported as a Flexner W. Subsequent biochemical studies demonstrated that it, also, was a probable paracolon. On first isolation this culture was anaerogenic in triple and single sugars, but it fermented glucose, maltose, and mannitol with production of acid and gas, upon transfer. The organism failed to ferment lactose, sucrose, dulcitol, and salicin in twelve days and did not utilize citrate or hydrolyze urea. It was indol positive, nonmotile, and reduced trimethylamine oxide to trimethylamine. Absorption studies with 8255 and Flexner W organisms and their antisera demonstrated that 8255 culture has a minor antigenic

relationship for Flexner W. Agglutinins for 8255 and Flexner W organisms were not present in the serum of the rabbit used for production of 8255 immune serum.

Also included in the total of 315 organisms are two paradysentery cultures which could not be identified by slide agglutination technique. One reacted in polyflexner antiserum but failed to agglutinate in the six monovalent sera. Although it was a typical paradysentery organism biochemically, and smooth in phase, it apparently possessed no type-specific antigen. This strain was classified as Flexner Y on the basis of its 3,4 group antigens. The second paradysentery culture was typical biochemically and unstable in saline suspension upon first isolation. After rapid transfer it was agglutinated in polyflexner antiserum by the tube method. No type determination was made.

Only one of the more exotic Shigella was identified in the study of 315 cultures. This organism did not react with the absorbed sera used routinely and was, therefore, put aside for biochemical tests. Of the carbohydrates, glucose, lactose, sucrose, maltose, mannitol, xylose, and dulcitol, it fermented glucose only in twenty-four hours. In ninety-six hours, maltose was fermented weakly. Its other characteristics were those of a Shigella. Of the absorbed sera prepared against mannitol-negative Shigella, it was agglutinated only in Sachs Q771 serum. An antiserum prepared against our strain 7005 agglutinated a stock strain of Q771 to approximately the titer of the homologous organism. Reciprocal absorption tests demonstrated that 7005 and the stock strain possessed similar antigens.

Since Sachs Q771 strains have been reported infrequently in this country,[15, 16] the source was of interest. Isolation was made from the fecal specimen of a 7-year-old Mexican child hospitalized with dysentery.

The identification of culture 8255 as a Flexner W was a definite error in classification due, unquestionably, to the use of inadequately absorbed W serum. A second error, also brought about by reliance on serologic identification alone, must be viewed in a different light. Among the 159 organisms classified as *Sh. sonnei* was a culture which gave atypical reactions biochemically. This culture, C-27, was found to be motile and anaerogenic, fermenting glucose, lactose, and maltose of the commonly used carbohydrates. By absorption studies it was found to possess the major antigen of *Sh. sonnei,* phase I, as defined by Wheeler and Mickle.[17] A description of C-27 will be made in detail in a separate publication since this organism poses an interesting problem in classification.

Biochemical Tests.—

Flexner Types: All of the sixty-five Flexner organisms typed with absorbed sera were typical in their fermentation reactions, fermenting glucose and mannitol, or glucose, maltose, and mannitol. None fermented xylose. The majority produced indol.

Boyd 103: Boyd 103 types were irregular in their reactions. Six strains from the same source fermented glucose only of the carbohydrates used routinely. Mannitol was not fermented after prolonged incubation; indol was formed by

all six strains. Antisera were produced against two of the cultures and absorption studies were carried out with stock antisera and stock cultures of Boyd 103. The mannitol-negative strains were able to exhaust the agglutinins of a Boyd 103 stock antiserum for both stock cultures and mannitol-negative strains. The same results were obtained when sera for the mannitol-negative Boyd 103 types were absorbed with stock cultures.

Two of the eight Boyd 103 types identified were typical in their reactions, fermenting glucose and mannitol in twenty-four hours. One strain produced indol and the other did not.

Boyd 88: All but three of the fifty-two Boyd 88 types classified serologically were from a single outbreak, and, as might be expected, the forty-nine strains from the outbreak were fairly similar in biochemical characteristics. All fermented glucose and mannitol promptly; the majority fermented maltose in six or seven days, whereas dulcitol was attacked slowly by most strains and a few did not ferment the carbohydrate in fourteen days.

None of the fifty-two strains produced indol. One strain, not from the outbreak, fermented glucose and mannitol only. The irregularity of fermentative reactions by organisms of the Boyd 88-Newcastle-Manchester series has been noted by numerous investigators[5, 18, 19] and has been attributed as regards gas production to the brand of peptone used as a base for fermentation media.[20] Two nonmotile, gas-forming cultures were examined by us which were identified serologically as type Boyd 88. Both appeared anaerogenic on triple sugar medium in twenty-four hours but produced a small amount of gas in forty-eight hours. Both fermented glucose and mannitol promptly, eventually producing a considerable bubble of gas in the Durham vials. Neither culture fermented dulcitol in eight days. Absorption studies demonstrated that both organisms possessed the major antigen of type Boyd 88.

Shigella sonnei: With the exception of culture C-27, the organisms identified as *Sh. sonnei* were typical in their reactions during a short period of incubation. All fermented glucose, maltose, and mannitol in twenty-four hours; none fermented xylose or produced indol. Prolonged incubation of the Sonne cultures was not made to obtain reactions in lactose and sucrose.

Shigella alkalescens: Stuart and associates[21] have pointed out that the antigenic and biochemical limits to the species *Sh. alkalescens* are difficult to fix. They report a study of 141 cultures corresponding to the classical description of *Sh. alkalescens* and forty-two cultures intergrading biochemically from *Sh. alkalescens* to *Escherichia coli* which possessed some or all of the antigenic fractions of *Sh. alkalescens.* It would appear that the identification of this species according to the strict definition is impossible by serologic means alone.

In order to discover what had been labeled as *Sh. alkalescens* by the slide agglutination test, strains isolated by us were examined in a wide range of carbohydrates and were grown on Simmons' citrate, urea medium, Voges-Proskauer broth, and semisolid motility agar. The majority of the cultures were typical in reaction, fermenting glucose, maltose, mannitol, and dulcitol, forming indol, and failing to grow on citrate or to produce acetyl-methyl-carbinol. They

were nonmotile. There were ten exceptions which were atypical in reaction with the difference that they grew on citrate in three to five days and were motile.

Shigella ambigua: The one *Sh. ambigua* culture identified serologically was typical in its biochemical reactions and was nonmotile.

Tube Agglutination: The 307 organisms classified as Shigella by slide agglutination technique were agglutinated to complete or moderate titer with appropriate antisera, with but few exceptions. An occasional strain was found to be partially dissociated by the time the tube method was employed and required transfer before a repeat test. Boyd 88 strains were not agglutinated strongly in the polyflexner serum employed for the tube agglutination titer, the average end point being 1:320. In contrast, Flexner strains were agglutinated in a dilution of 1:1280 to 1:5120 in the same serum. The average end point for *Sh. sonnei* strains in specific serum was a dilution of 1:5120. All of the *Sh. alkalescens* strains were agglutinated strongly in unabsorbed specific serum.

DISCUSSION

The rapid identification of Shigella by the use of absorbed sera has become an established procedure in some laboratories, since there are definite advantages in its employment in the slide technique over the tube method with unabsorbed sera. It is now a question with those who have adopted the slide agglutination technique of how far shall we go in accepting the results of serologic classification. Shall we report the identity of an organism as Shigella solely on the results of serologic identification with highly absorbed sera, or shall we combine serologic identification with biochemical confirmation studies to arrive at the identity of a dysentery-like organism?

The first course is attractive because of the rapidity with which a report may be made to a physician. Instead of a period of seventy-two to ninety-six hours between receipt of a specimen and a report on the Shigella present, there is the possibility of a report in forty-eight, or even twenty-four, hours if typing is made direct from an isolation plate. To offset the advantage of speed there is the possibility of an error in identification.

The chances of error are not great but they are definite. There is first the possibility that all cross-reacting agglutinins have not been absorbed from sera. An error due to inadequate absorption of Flexner W antiserum is reported in the present study. There is the possibility of reaction between absorbed serum and its specific antigen existing in a different species, as in the case of culture C-27, resulting, nevertheless, in an incorrect identification.

Just what the significance of an organism such as C-27 is in the scheme of the Enterobacteriaceae is uncertain; one would hesitate to classify this culture as *Sh. sonnei* from our present imperfect knowledge of the species and of organisms related to it antigenically. Wheeler and Stuart[16] have described a motile paracolon strain with the complete somatic antigen of Sachs type Q771, and it seems possible that similar relationships between paracolon and Shigella organisms will be discovered in the future. Whether these related but atypical

organisms will be numerous enough to cause frequent errors in classification seems very doubtful. The paucity of reports in the literature would indicate that such cultures are few.

Should classification in the present study of ten motile, citrate-positive strains as *Sh. alkalescens* be considered as further error due to serologic methods? According to the present limits of the species, yes. It is evident, however, from the work of Stuart and co-workers[21] that a new conception of the species may be realized and that classification according to the present limits may be changed.

Since *Sh. alkalescens* isolated from feces is not reported to the physician by our laboratory, any discussion of error in the classification of the motile, citrate-positive organisms is academic. It seems probable that many of these cultures have been, and are being, classified as *Sh. alkalescens* on the basis of conventional methods of identification. Motility is usually sluggish and is noticeable in semisolid medium only after twenty-four to thirty-six hours' incubation, while the reaction on citrate is slow, alkalinity appearing in three to five days.

In view of the results in the present study, it would appear that the chances of error in identifying the pathogenic Shigella by use of highly absorbed sera are slight. If we except from discussion the controversial issue of the limits to the species *Sh. alkalescens*, our rate of error was two out of 284 specimens, or approximately 0.7 per cent. When one considers methods of identification of several years ago, the results by the rapid method look very good indeed. How then would the mannitol-negative Boyd 103 strains have been classified? Would a report on either the Boyd 103 or the aerogenic Boyd 88 types have been made within a reasonable period of time in order that they might be of use to a physician?

A contrast of past and present methods in which present methods appear to advantage should not overlook the fact that the latter are subject to occasional errors. Therefore, at the completion of this study, our recommendation was as follows: A provisional report on the identity of Shigella-like organisms should be made, based on the outcome of the slide agglutination test. In our opinion the value of a rapid report to the physician justifies this procedure. Confirmatory motility and biochemical studies should continue to be made on each strain examined. This should be done both for confirmation of serologic results and to add to our knowledge of irregular biochemical reactions among the Shigella.

SUMMARY

1. Methods for production of highly absorbed Shigella antisera for use in the slide agglutination test are presented.

2. A total of 315 cultures which appeared dysentery-like on triple sugar agar was examined by slide agglutination technique with absorbed sera. Of these, 307 were accepted as Shigella on the basis of their reactions in absorbed sera. One of the 307, identified as *Sh. sonnei*, was found to be atypical in fermentation reactions and motile, yet it possessed the major antigen of *Sh. sonnei*.

Of thirty-two cultures identified serologically as *Sh. alkalescens,* ten were atypical in that they utilized citrate slowly and were motile. The remainder of the 307 cultures were confirmed as Shigella by means of biochemical and tube agglutination tests.

3. One Sachs type Q771 strain was identified.

4. Rapid identification of two paradysentery strains could not be made because of "rough" phase in one culture and lack of specific antigen in the other.

5. Of five paracolon strains which reacted with polyvalent Flexner antiserum, four were eliminated as pathogens because of failure to react with monovalent serum; the fifth was incorrectly identified as Flexner W.

6. It was concluded that immediate reports on the identity of Shigella are justified when they are based on the rapid slide agglutination test, provided antisera used in the test have been highly absorbed. All identifications should be followed by tests of motility and biochemical studies.

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THE SEROLOGIC RESPONSE IN MURINE TYPHUS AS MEASURED BY THE WEIL-FELIX, RICKETTSIAL COMPLEMENT FIXATION, AND RICKETTSIAL AGGLUTINATION REACTIONS

CARL T. NELSON, M.D.
NEW YORK, N. Y.

SINCE its introduction in 1916, the Weil-Felix test[1] has been a valuable aid in the diagnosis of rickettsial diseases in human beings. This reaction, based on the development of nonspecific agglutinins for the O or nonmotile variants of certain strains of Proteus X, has been widely used for diagnostic purposes because of its simplicity and accuracy in detecting rickettsial infections. In common with other nonspecific serologic reactions, however, the Weil-Felix test has limitations which, if not restricting its usefulness, often render its interpretation difficult. For example, it is now generally agreed that the test does not distinguish between typhus and the rickettsial diseases of the spotted fever group. This is of some importance in regions such as the Southern United States where murine typhus and Rocky Mountain spotted fever are both endemic. Moreover, the sera of normal persons who have never been exposed to rickettsial infection often contain low-titer agglutinins for Proteus X strains. Consequently it is usually not safe to draw diagnostic conclusions from the results of a single Weil-Felix test, even if the titer exceeds a level commonly regarded as significant. Another source of difficulty in assessing the significance of the Weil-Felix reaction may arise from the fact that agglutinins for Proteus X strains sometimes occur as a result of infection with *Proteus vulgaris* and *Proteus mirabilis*.[2]

With the development of improved methods for cultivating rickettsiae in relatively large amounts, it has become feasible to employ purified rickettsial suspensions as antigens in the serologic diagnosis of typhus and related diseases. When such antigens are used in complement fixation or agglutination tests they afford convenient means for diagnosing rickettsial infections. In addition, their greater specificity permits a differentiation between the various rickettsial diseases which give overlapping results with the Weil-Felix test.[3-14] The antigens most commonly used at the present time are prepared by growing rickettsiae in the yolk sac of the developing chick[15] or in the lungs of rodents.[16, 17]

It is evident from data published thus far that there has been little occasion for studying the comparative effectiveness of suspensions of rickettsiae and Proteus OX strains in the early serologic diagnosis of murine typhus. As Felix has pointed out,[18] murine typhus is essentially a sporadic disease with a relatively low fatality rate, and patients usually do not come under observation soon enough to permit an adequate investigation of the pattern of serologic

From the Department of Dermatology, College of Physicians and Surgeons, Columbia University.
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response in the early stages of infection. The studies reported here were undertaken primarily to investigate the course of antibody development in patients with murine typhus by comparing the results of the Weil-Felix test with the specific rickettsial agglutination and complement fixation reactions. It was hoped that in this way it might be possible to evaluate more clearly the relative usefulness of the murine rickettsial agglutination and complement fixation tests in the early diagnosis of the disease.

METHODS AND MATERIALS

For the purposes of this study, multiple samples of blood serum were collected over a period of several weeks from patients with clinical evidence of typhus infection. In almost all instances these patients were residents of communities in the Southeastern United States where murine typhus is endemic. Efforts were made to obtain samples of serum at intervals of two to four days during the first three weeks of illness and at seven or eight days for the next five weeks. Thereafter, whenever possible, serum was collected once each month until a period of 150 days following the onset of illness had elapsed. In many cases, however, the patient could not be observed beyond the initial eight-week period. Although it was thus possible to obtain accurate data on the development of antibody levels, the persistence of titers in these cases could only be partially studied.

Sera collected in this manner were sent to a central laboratory where agglutination and complement fixation reactions with murine rickettsial antigens* as well as agglutination tests for Proteus strains OX 19, OX 2, and OX K were performed with each specimen. Sometimes complement fixation tests were also carried out with Rocky Mountain spotted fever rickettsial antigen. The serum specimens were tested as they were received at the central laboratory. In addition, the unused portions of successive sera from any one patient were stored at 4° C. and later retested simultaneously by each method. This was done to safeguard against possible variations in the reactivity of the antigenic suspensions. Only those patients who gave an accurate history regarding date of onset of symptoms and in whom it was possible to trace the progressive development of antibody titers during illness have been included in this report. Of the thirty-four nonfatal cases of murine typhus originally comprising this series, twenty-two could be studied sufficiently closely to yield accurate and comparable data.

The Proteus OX antigens employed in the Weil-Felix tests were alcohol-treated suspensions prepared and standardized according to Bridges' modification of Bien's method.[19, 20] Prior to use, the concentrated suspensions were adjusted to a density approximating the McFarland nephelometer standard No. 3. For the agglutination tests, 0.5 c.c. amounts of suspension were added to 100 by 13 mm. round-bottomed tubes containing equal volumes of the patient's serum which had been made up in serial twofold dilutions ranging from 1:10 to 1:640. All tests were controlled with positive and negative sera. The tubes were centrifuged at 2,000 r.p.m. for seven minutes and then read macroscopically after shaking slightly to dislodge the particles from the bottom of each tube. The highest serum dilution showing any degree of agglutination was recorded as the titer. The reaction was arbitrarily considered to be positive when agglutination occurred in an initial serum dilution of 1:80 or more or when a fourfold increase in titer over a previous specimen had been observed.

Rickettsial agglutination tests were performed with antigens obtained from infected yolk sacs of developing chick embryos.[15] The rickettsial suspensions were prepared in the manner recommended by Fitzpatrick,[14] using the ether-extraction method of purification. Such suspensions were found to be relatively stable, retaining their original specificity and sensitivity for a period of at least six months when stored at refrigerator temperatures. Serum specimens for examination were diluted serially from 1:20 to 1:160 and tested by the slide agglutination technique previously described.[14] Positive and negative control sera were

*The murine rickettsial antigens for agglutination tests were provided by the courtesy of Miss Florence K. Fitzpatrick, Sharp & Dohme, Inc., Glenolden, Pa. The rickettsial antigens employed in the complement fixation studies were supplied through the kindness of Dr. Ida A. Bengtson, National Institute of Health, Bethesda, Md.

included in each test. The slides were placed in the incubator at 40° C. for five hours and then stored at 4° C. overnight. Preliminary macroscopic readings were made after the slides had remained in the incubator for one and five hours, respectively. A final reading was made with the low-power objective of the microscope after overnight refrigeration. The test was recorded as positive only when complete agglutination was observed in an initial serum dilution of 1:40 or more. When agglutination occurred it usually was visible to the unaided eye within a period of one hour. In reading results the slide test presented none of the difficulties encountered by van Rooyen and associates[12] with rickettsial agglutination reactions in test tubes.

The antigens used for the complement fixation tests were also prepared from rickettsiae grown in the yolk sac of embryonated hen eggs.[15] The rickettsial suspensions were made up and standardized by the method employed by Bengtson.[21] Serum samples from each patient were inactivated at 56° C. for one-half hour and then diluted serially from 1:4 to 1:512. The technique of the complement fixation test was that described by Bengtson,[21] using the customary reagent controls. Each day's tests were controlled also with positive and negative sera. The degrees of fixation were recorded as 4, 3, 2, and 1 plus, and trace. In this test a reaction of 3 or 4 plus in a serum dilution of 1:8 or higher was considered to be positive.

RESULTS

The results of the serologic studies in the twenty-two patients with murine typhus included in this series are summarized in Table I. Generally speaking, in most of these patients the Weil-Felix and rickettsial agglutination tests became positive earlier in the course of illness than did the rickettsial complement fixation reaction. Sometimes the Weil-Felix became positive slightly sooner than the rickettsial agglutination test; occasionally the reverse was true. For the most part, however, these reactions differed very little in this respect. In only one case was the rickettsial complement fixation test found positive before the other two.

TABLE I. EARLY SEROLOGIC RESPONSE, BY DAY OF DISEASE, IN TWENTY-TWO PATIENTS WITH MURINE TYPHUS

	NUMBER OF PATIENTS SHOWING A POSITIVE TITER BY		
DAY OF DISEASE	WEIL-FELIX	MURINE RICKETTSIAL AGGLUTINATION	MURINE RICKETTSIAL COMPLEMENT FIXATION
4	0	0	0
6	1	1	1
8	2	2	2
10	9	9	6
12	16	15	7
14	20	19	9
16	21	19	12
18	21	20	13
20	22	20	14
22	22	22	15

During the first week of illness all three tests seemed to be equally ineffective in establishing a serologic diagnosis of murine typhus. In the second week of illness the Weil-Felix and rickettsial agglutination tests were usually positive and were clearly superior to the complement fixation reaction in detecting the disease. Even three weeks after onset, when the two agglutination tests were positive in all instances, the complement fixation reaction was still negative in nearly one-third of the cases. In one patient the presence of complement-fixing

antibody was not demonstrated until six weeks after illness began. Further data, comparing the per cent of positive Weil-Felix, rickettsial agglutination, and rickettsial complement fixation reactors by day of disease, are shown in Fig. 1.

These findings suggest that the Weil-Felix and rickettsial agglutination tests are more valuable than the rickettsial complement fixation reaction in the early serologic diagnosis of murine typhus. The rickettsial agglutination reaction, in turn, is superior to the Weil-Felix test in that it is more specific and permits a differentiation to be made between the murine and spotted fever types of infection. In view of this one may conclude that the rickettsial slide agglutination test, as performed in this work, is a superior serologic method for detecting murine typhus in the early stages of the disease.

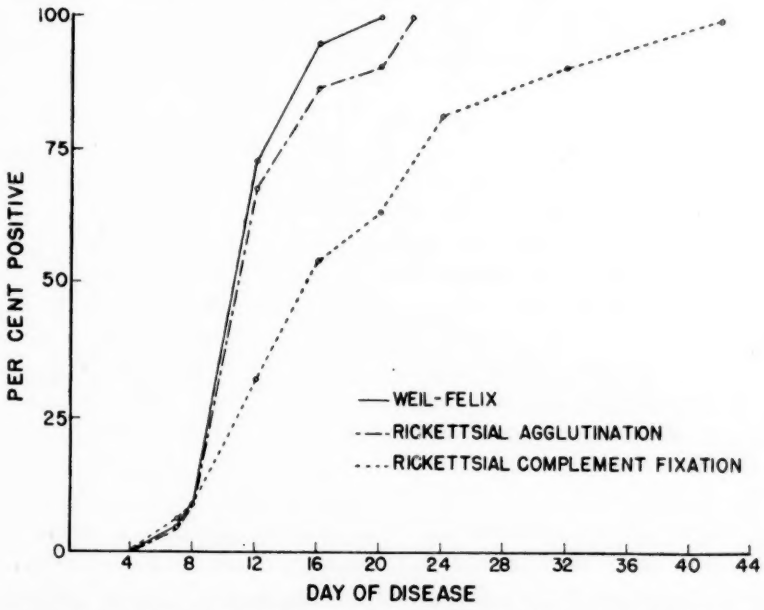

Fig. 1.—Per cent of positive Weil-Felix, rickettsial agglutination, and rickettsial complement fixation reactors, by day of disease, in twenty-two cases of murine typhus.

Additional information gained in the course of these studies indicates that once the rickettsial complement fixation reaction becomes positive it remains so for a relatively long period of time. In this respect it does not appear to differ significantly from the rickettsial agglutination test. Although the persistence of antibody levels in these patients could not be studied completely, it seems clear that the Weil-Felix is the most evanescent of the three reactions and frequently becomes negative eight to twelve weeks after the onset of symptoms.

DISCUSSION

According to Felix,[18, 22] the majority of cases of louse-borne typhus and many cases of murine typhus can be diagnosed by the OX 19 reaction during the first week of illness. This has been denied by other investigators[12, 23] who

point out that significant agglutinin titers for rickettsiae and Proteus OX 19 usually are not attained by such patients until early in the second week of disease. In so far as murine typhus is concerned, the data presented here indicate that in most cases the disease cannot be diagnosed by serologic methods until after the eighth day of illness.

From time to time in the past, certain workers have postulated that the pattern of agglutinin formation in cases of typhus is related to the clinical course of the disease.[18, 24] Generally speaking, patients with cases of moderate severity are said to respond with high titers, while those with most severe cases, including those who succumb to the infection, have very low titers. In this small series of patients, little or no relationship between the severity of illness and the magnitude of antibody response was found. While this work was in progress it was possible to observe three patients with uncomplicated murine typhus which terminated fatally. In each of these the agglutinin and complement-fixing titers reached high levels prior to death. Although the number of patients studied does not permit one to draw definite conclusions, it seems that the antibody levels attained in murine typhus are subject to wide individual variations and that if an inverse relationship exists between clinical severity and the height of antibody response it must be a tenuous one.

The specificity of rickettsial agglutination and complement fixation reactions already has been demonstrated conclusively by several investigators.[4, 8, 10, 12-14, 17, 25] In the absence of previous rickettsial infection or vaccination, sera from normal persons and individuals suffering from nonrelated febrile illnesses almost never contain antibodies for rickettsial antigens. This was amply confirmed during the course of these studies. On the other hand, some degree of cross agglutination and complement fixation with murine, classic, and spotted fever antigens may be expected to occur in sera from patients with typhus and spotted fever. When the rickettsial suspensions are properly standardized, however, the titer for the infecting strain is usually significantly higher than the heterologous antibody levels. Thus a serologic differentiation may be made between diseases of the typhus and spotted fever groups by means of the agglutination and complement fixation tests.

Plotz and associates[26] have recently indicated that the rickettsial agglutination reaction is less effective than the complement fixation test in differentiating between typhus and Rocky Mountain spotted fever. These investigators, using an unspecified macroscopic agglutination technique, found that the sera from thirteen patients with Rocky Mountain spotted fever gave considerable agglutination with murine and classic rickettsiae, whereas no cross reactions for the typhus antigens occurred in the rickettsial complement fixation test. During the present study there was an opportunity to observe the serologic response in three patients with Rocky Mountain spotted fever. In two of these no cross agglutination with murine typhus antigen was noted in the slide agglutination test. In the third patient partial agglutination in low titer (1:40) occurred with murine rickettsiae, but this titer remained stationary throughout the disease. Sera from all three patients, however, gave slight to moderate cross reactions with murine

rickettsial suspensions in the complement fixation test. While it is obviously impossible to generalize from scattered observations of this sort, such findings tend to reaffirm the value of the slide agglutination test used in these studies for the differentiation of rickettsial diseases. Moreover, from a clinical standpoint, these results emphasize that the more elaborate complement fixation reaction probably possesses no differential diagnostic virtues which are not also shared by the simple slide agglutination test.

SUMMARY

1. An investigation of the serologic response in twenty-two cases of murine typhus has shown that the Weil-Felix and rickettsial agglutination reactions usually become positive earlier than the rickettsial complement fixation test in this disease.

2. Because of its specificity and sensitivity, the rickettsial slide agglutination test employed in these studies is particularly useful in the early serologic diagnosis of murine typhus.

3. In so far as the detection of murine typhus in its acute stages in man is concerned, the relatively simple rickettsial slide agglutination test is fully as effective as the more elaborate complement fixation reaction.

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INFLUENZA B IN NEEDHAM, MASSACHUSETTS, DECEMBER, 1945

Serologic Studies in Cases and in Family Contacts

Maxwell Finland, Mildred W. Barnes, Manson Meads, Edwin M. Ory, and A. Daniel Rubenstein
Boston, Mass.

ATTENTION was first called to the possibility of a sizable outbreak of influenza in Needham, Mass., by an abrupt rise in the number of absentees in the Junior High School during the second week of December, 1945. Through the cooperation of the local school and health authorities and of many of the residents of the community, an opportunity was afforded to study certain aspects of this outbreak. The present paper includes a report of serologic studies made in a limited number of cases and family contacts. The epidemiologic observations are reported elsewhere.[1]

MATERIALS AND METHODS

Collection of Materials.—Visits were made to the Junior High School and to the homes of a number of pupils who were acutely ill with influenza between Dec. 13 and 16. Throat washings were obtained at this time from several individuals who were still acutely ill with the symptoms of influenza and with a fever of 101° F. or higher. At the same time samples of venous blood for serologic studies were obtained aseptically from individuals with acute influenza and from members of their families, including both persons with influenza and apparently healthy contacts. Clinical histories were also obtained at this time. A second visit was made to each of the same families between Dec. 29, 1945, and Jan. 2, 1946. At that time blood was again drawn from patients and contacts and a history was obtained of any illnesses that may have occurred during the intervening period. As far as could be ascertained, none of the patients or contacts had received inoculations of influenza vaccines.

Clinical Findings.—The clinical features of the cases of influenza were quite characteristic and consisted of fever (100 to 104° F.), prostration, eyeball pain, generalized aching, some cough, and occasionally a mild sore throat. There was usually slight conjunctival injection and supraorbital edema. A few scattered râles were heard in the lungs of some of those individuals who were examined. The severity of the illness was rated as 1 to 3 plus, according to the degree of prostration, height and duration of fever, and the severity of the systemic symptoms. Four adult contacts of patients with influenza had slight fever and predominantly gastrointestinal symptoms which had been called "in-

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, the Department of Medicine, Harvard Medical School, and the Massachusetts Department of Public Health.

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testinal flu." There were four other contacts, one a cafeteria worker at the school and three parents of individuals with influenza, who were afebrile and had symptoms characteristic of a common cold, with coryza predominating.

Antigens.—The antigens used in the serologic tests included the PR8 strain of influenza A, the Lee strain of influenza B, strains WC and MF isolated from throat washings obtained on Dec. 14 from patients with acute influenza among the Thorndike Laboratory staff who had no contact with the Needham cases, and strain MB isolated from throat washings on the first day of illness from one of the patients in the Needham outbreak (patient 6 in Table I). The PR8 and Lee viruses were originally obtained from Dr. Thomas Francis, Jr., and the others were isolated by amniotic inoculation and passage and were subsequently transferred through more than twenty allantoic passages. Each was concentrated from a pooled harvest of allantoic fluid by elution from the embryonic erythrocytes according to the method of Francis and Salk[2] and kept in a CO_2 icebox at about –70° Centigrade. Details of the isolations of influenza strains during this epidemic will be presented elsewhere.

Serologic Tests.—The sera were kept in an electric refrigerator (deep freeze) at –20° C. in rubber stoppered Pyrex tubes and rapidly thawed each time before any was withdrawn for the tests. All serologic tests were carried out in two-fold dilutions of serum and saline beginning with 1:4. Paired sera from each person always were tested at the same time, usually with two or more viruses. A majority of the tests were repeated on two or more separate occasions. At least on one occasion in most cases, dilutions of the sera made up at one time were run simultaneously with three or four of the viruses in both agglutinin inhibition and complement fixation tests. The agglutinin inhibition tests were set up essentially as described by Hirst.[3] Chicken blood for these tests was obtained from a local slaughterhouse and the blood from three or more chickens was pooled each time. The details of the complement fixation test were similar to those described by Enders and associates[4] for the mumps virus. Titers were recorded as the reciprocal of the greatest initial dilution of serum before adding other reagents. The end point in the Hirst test was read at the dilution in which erythrocyte agglutination was completely absent (0) or estimated up to 25 per cent (+). In the latter event the titer was arbitrarily reduced by one-half of the value of the preceding interval. (Example: Complete inhibition in 1:64 and plus agglutination in 1:128; titer is 128 less 32 or 96.) In the complement fixation test the end point was graded 1 to 4 plus, the former representing an estimated 75 per cent hemolysis and the latter complete fixation, that is, no hemolysis. The 1, 2, and 3 plus readings were reduced ¾, ½, and ¼, respectively, of the value of the preceding interval. Examples: No hemolysis (4 plus) in 1:64 and complete hemolysis (0) in 1:128; titer is 64. No hemolysis (4 plus) in 1:64 estimated 25 per cent hemolysis (3 plus) in 1:128, and complete hemolysis (0) in 1:256; titer is 128 less ¼ of 64 or 112.) The titers of serum which showed no antibody in the initial dilution (1:4) were arbitrarily given the value of 2.

RESULTS

Virus Isolations.—Attempts to isolate a virus from the throat washings obtained from patients with acute clinical influenza in the Needham outbreak were unsuccessful in most instances. The washings had been kept chilled during transit and stored in sealed Pyrex tubes in a CO_2 icebox as soon as they reached the laboratory. Unfiltered washings with 150 to 500 units of penicillin added were inoculated. Various combinations of amniotic and allantoic passages in eggs with or without mouse passages were used. In some instances evidence suggesting the possible presence of influenza virus was obtained in the course of some of the early passages in the embryonated eggs by the hemagglutination of the embryonic erythrocytes, but subsequent passages failed to establish the presence of a virus in most of them. Successful isolations of influenza viruses from this season's cases had been obtained only from the three cases mentioned at the time the present studies were being carried out. The three strains were readily identified serologically as type B influenza and were used in the present studies for comparison with the Lee strain.

Cases Studied.—Included in the serologic study are eleven families and two isolated cases, a total of thirty-nine persons. In each family there was at least one with typical symptoms of clinical influenza of moderate severity and from one to four others, either patients or healthy family contacts, from whom sera for serologic studies were obtained at the same time. The two isolated cases included a pupil and a cafeteria worker at the Junior High School. The influenza antibody titers in each of these individuals are shown in Table I, together with the date of onset and a rough estimate of severity in the cases of clinical influenza. Each titer represents the average for all the tests done with the virus and serum concerned. Patients in whom the symptoms were essentially those of a mild common cold with little or no fever and with coryza as the only significant feature are listed under Severity as *C;* those with low grade fever and diarrhea as the outstanding manifestation are designated as *D* in the same column in Table I.

Serologic Results in Typical Cases of Influenza.—

Influenza A Antibodies: There were nineteen characteristic cases of clinical influenza, and the symptoms in each instance began between Dec. 9 and 15. There were three such cases in each of two families, two in each of three families, and six that were the only typical cases among the persons studied in their families; there was one isolated case of clinical influenza with no family contacts included in the study. In none of these nineteen cases was there any significant rise in titer of antibodies against the PR8 strain of influenza A by either the agglutinin inhibition or complement fixation test.

The level of the antibody titers obtained with the PR8 strain by the tests employed are worth noting. A titer of 32 or higher was obtained in only one of the sera (the convalescent serum of Patient 7) by the complement fixation test, whereas titers ranging from 64 to 384 were obtained by the agglutinin inhibition test in both of the sera from six individuals and in the acute serum of a seventh person. The low titers were not the result of poor sensitivity of the

TABLE I. RESULTS OF SEROLOGIC TESTS

PATIENT	FAMILY	SEX	AGE	CLINICAL INFLUENZA: DATE OF ONSET	CLINICAL INFLUENZA: SEVERITY	DATE OF SERUM	AVERAGE TITER OF INFLUENZA ANTIBODIES: PR8 AGGLUTININ INHIBITION	PR8 COMPLEMENT FIXATION	LEE AGGLUTININ INHIBITION	LEE COMPLEMENT FIXATION	WO AGGLUTININ INHIBITION	WO COMPLEMENT FIXATION	MF AGGLUTININ INHIBITION	MF COMPLEMENT FIXATION	MB AGGLUTININ INHIBITION	MB COMPLEMENT FIXATION
1	F	M	15	Dec. 9	++	Dec. 14	80	16	51	48	32	19	24	28	24	32
				Dec. 30		Dec. 30	72	12	597	363	384	216	256	320	384	448
2		F	13	--	-	Dec. 14	48	3	14	6	16	10	12	10	32	6
						Dec. 30	48	3	24	15	16	28	16	28	64	24
3		F	49	--	-	Dec. 14	24	3	11	11	16	14	8	14	4	10
						Dec. 30	32	3	13	9	16	14	16	14	4	12
4		M	57	--	-	Dec. 14	12	10	8	15	4	14	3	14	4	20
						Dec. 30	12	12	8	20	4	20	4	14	2	14
5		M	75	--	-	Dec. 14	6	2	6	9	4	16	8	16	3	5
						Dec. 30	6	2	4	12	4	28	8	28	6	20
6	Ba	F	12	Dec. 13	+++	Dec. 13	28	3	9	2	4	2	3	2	6	2
						Jan. 2	40	3	172	107	96	64	16	56	128	112
7		F	10	Dec. 9	++	Dec. 15	160	24	6	2	3	10	3	10	12	5
						Jan. 2	192	40	384	256	128	576	128	512	512	448
8		F	9	--	-	Dec. 15	384	28	6	4	2	5	6	2	4	2
						Jan. 2	128	28	6	3	2	10	12	3	3	3
9		F	42	Dec. 8	C*	Dec. 15	8	2	6	4	2	4	2	3	6	3
						Jan. 2	8	2	6	7	2	5	2	3	3	5
10	D	M	14	Dec. 10	+++	Dec. 13	24	9	20	14	8	12	24	16	24	16
						Dec. 29	24	6	544	512	256	192	256	448	192	512
11		F	40	--	-	Dec. 15	16	12	8	8	2	8	6	8	8	10
						Dec. 29	6	6	6	16	4	14	6	16	16	14
12		M	43	Dec. 5	D†	Dec. 15	32	16	28	26	2	96Ac‡	8	20	64	24
						Dec. 30	24	16	28	20	3	20	8	16	64	24
13		F	65	Dec. 15	+++	Dec. 29	3	3	192	224	128	192	128	192	192	192
14	Mc	M	13	Dec. 7	+++	Dec. 14	24	4	80	52	64	64	32	96	192	112
						Dec. 30	24	4	80	60	64	72	32	80	128	56
15		M	14	Dec. 12	++	Dec. 14	48	4	10	2	--	--	--	--		
						Dec. 30	48	4	320	256	128	112	48	80		
16		M	14	Dec. 13	+++	Dec. 14	8	2	12	2	--	--	--	--		
						Dec. 30	6	3	512	256	192	192	96	112		
17		F	44	--	-	Dec. 14	12	3	20	32	2	32	8	24	32	20
						Dec. 30	12	6	20	72	2	32	8	24	24	20
18	Mo	F	17	Dec. 10	+++	Dec. 13	48	11	6	10	2	10	6	10	4	12
						Dec. 30	48	11	6	44	4	32	16	40	4	48

19		M	44	Dec. 26	D	Dec. 16	16	12	7	12	4	10	8	8	4	20
						Dec. 30	16	16	8	20	4	12	12	12	4	24
20		F	40	--	-	Dec. 16	16	7	9	17	4	16	3	12	4	24
						Dec. 30	16	8	12	32	4	20	3	16	4	24
21	H	F	14	Dec. 11	+++	Dec. 14	16	10	3	6	2	2	2	3	2	5
						Dec. 29	20	8	4	8	16	12	16	10	2	7
22		F	38	Nov. 1	C	Dec. 14	24	10	6	5	6	10	6	18	2	5
						Dec. 29	32	12	6	5	6	7	7	14	2	5
23		M	40	--	-	Dec. 14	8	6	16	7	12	10	8	12	4	20
						Dec. 30	12	6	12	11	12	12	12	12	4	12
24	W	M	10	Dec. 12	++	Dec. 16	3	2	10	2	3	2			24	3
						Dec. 30	3	2	10	2	4	2			24	3
25		F	14	Dec. 13	++	Dec. 16	128	10	48	6	16	7	16	7	48	12
						Dec. 30	128	10	32	6	16	7	16	7	64	20
26		M	50	Dec. 10	+	Dec. 16	64	10	20	13	5	12	8	14	24	12
						Dec. 30	64	8	20	12	5	10	8	14	24	10
27	V	F	11	Dec. 15	+++	Dec. 15	64	8	3	2	2	2	2	2	3	2
						Dec. 30	72	7	3	2	6	14	16	6	3	3
28		F	40	Dec. 10	C	Dec. 15	12	2	6	16	2	16	2	20	16	20
						Dec. 30	12	3	6	16	2	16	2	10	8	24
29		M	41	Dec. 12	D	Dec. 30	8	2	3	2	2	2	2	2	3	2
30		M	15	Dec. 8	+++	Dec. 13	64	16	12	12	--	--	--	--	8	8
						Dec. 29	32	16	512	256	320	256	128	224	256	512
31		F	44	--	-	Dec. 16	8	5	6	4	2	5	4	4	4	3
						Dec. 29	4	5	5	4	2	6	4	4	3	3
32		M	47	Dec. 6	D	Dec. 16	2	3	14	72	2	48			32	48
						Jan. 2	2	3	14	72	2	64			32	80
33	N	F	13	Dec. 14	+++	Dec. 16	20	30	7	21	2	2				
						Dec. 29	18	22	512	512	512	960				
34		F	36	--	-	Dec. 16	4	14	4	8	2	4	2	5	6	20
						Dec. 29	8	3	4	7	2	4	2	6	6	5
35		M	37	--	-	Dec. 16	8	10	5	5	2	3	2	3	8	3
						Dec. 29	8	10	6	3	2	3	2	3	8	3
36	Br	F	11	Dec. 10	+++	Dec. 15	48	4	16	15	16	24	16	24	8	20
						Dec. 29	48	4	128	160	128	256	128	256	96	320
37		F	46	Dec. 11	++	Dec. 15	12	2	10	6	2	7	6	8	8	14
						Dec. 29	12	3	24	44	16	32	32	64	32	128
38	P	F	46	Dec. 13	C	Dec. 14	48	4	8	5	2	5	2	5	6	2
						Dec. 29	40	3	9	5	2	6	2	10	6	3
39	T	M	13	Dec. 10	++	Dec. 13	24	14	6	4	2	20				
						Dec. 30	24	16	64	256	48	128				

*C, Common cold.
†D, Diarrhea and fever.
‡Ac, Anticomplementary.

strain, since high titers and sharp rises in titers to high levels were obtained with the same antigen by both tests in unvaccinated individuals and in several patients with influenza A which occurred late in January, 1946.[5]

Influenza B Antibodies: Significant rises in titer of influenza B antibodies were demonstrated with two or more of the four strains used in fourteen of these cases and the rises were quite marked in most instances. Two additional cases showed significantly high titers but no rises were demonstrated. In Patient 14 the initial blood was obtained on the seventh day after the onset of symptoms, and while no significant rise in titer of antibodies for any of the type B strains was demonstrated, the titers of these antibodies initially were comparable with or higher than the titers obtained with the convalescent phase sera in several other cases in which a fourfold or greater rise had been demonstrated. This suggests the likelihood that a maximum titer already had been reached in that case at the time when the initial blood was drawn. Twin siblings of this case each showed a marked rise in influenza B antibodies but the onset of their symptoms occurred five and six days later, respectively, and their initial titers were much lower. In Patient 13 only a single sample of blood was obtained fourteen days after the onset of symptoms and in that serum high titers of antibodies against all of the influenza B strains were obtained by both tests. These titers were comparable with the convalescent titers in cases exhibiting a marked rise, indicating that in this case too the high titers were probably the result of recent infection with influenza B.

Negative Serologic Responses: The three remaining typical cases of clinical influenza (Patients 24 to 26) showed no significant titers or rises in titers of influenza B antibodies. The patients were all members of Family W and were the only persons studied in that family. The initial sera in these cases were obtained between the third and sixth days after the onset of clinical influenza. The illness in these patients occurred during the peak of the outbreak and was considered to be of average severity in two and mild in the third. The antibody titers obtained with the Lee strain and with two or all three of the recently isolated strains of influenza B were low in almost every instance. The initial titers of antibodies for PR8 were moderately high in the agglutinin inhibition tests in two of these cases but the complement-fixing antibodies were of low titer for this strain in both instances. Both tests gave low titers with the PR8 strain in the third case.

Strain Differences: A few details concerning the influenza B antibodies in the typical cases of clinical influenza are worth noting. In every case in which a rise in titer of antibodies was demonstrated with the Lee strain, comparable rises in titer of antibodies also were demonstrated with the recently isolated strains. There were three cases, however, in which tests with the Lee strain failed to bring out a rise in titer of influenza B antibodies by either agglutinin inhibition or complement fixation or by both tests when such a rise was demonstrated with the recently isolated strains. The original titers of influenza B antibodies in these cases were all low. The sera in Patient 18 showed no rise in titer of agglutinin inhibiting antibodies for the Lee virus when tested on

three separate occasions; however, a significant rise in titer was demonstrated by the complement fixation tests simultaneously with the same virus, and dilutions of serum made up at the same time. Tests done with the WC and MF strains also showed two- to fourfold rises in titer in this case, but the results with the epidemic strain MB were similar to those obtained with the Lee strain. The sera in Patients 21 and 27 showed no rise in titer of antibodies for the Lee strain by either the agglutinin inhibition or complement fixation tests done on four separate occasions, but they showed appreciable rises by both tests done with the WC and MF strains. In neither of these two was any significant rise elicited with the MB strain either. There were no gross discrepancies between the results obtained in the same sera with the WC and MF strains by either agglutination inhibition and complement fixation tests, and the differences that were noted were quantitative and were usually only slight. The findings in these three cases suggest that the WC and MF strains are more closely related to each other than to the Lee and MB strains and also that the MB strain is more nearly like the Lee strain than are the other two recently isolated strains. Such a deduction, however, requires substantiation from more detailed antigenic studies before it is acceptable.

Cases Resembling Common Cold.—The four patients in whom fever was absent and the symptoms were considered to be those of the common cold are of interest. Three of them (Patients 9, 22, and 28) were mothers of families in which at least one child had clinical influenza associated with a significant rise in titer of antibodies for the type B viruses. The fourth (Patient 38) was an adult cafeteria worker at the Junior High School. The onset of symptoms in Patient 22 occurred early in November, 1945, but in the others the illness began at about the same time as in those patients with the typical course of clinical influenza. The sera of each were tested several times with the PR8 and Lee viruses and at least once with each of the three recently isolated strains. None of these four cases showed significantly elevated titers or rises in titers of antibodies for any of the viruses used. The initial titers, in fact, were similar to those obtained with the same viruses in the acute phase sera of cases of clinical influenza showing typical rises with the strains of influenza B.

Patients With Intestinal Manifestations.—The four patients in whom the symptoms were predominantly gastrointestinal are also of interest because of the possible relation of influenza virus infection to these symptoms. Each of these patients was the father of a family in which there was at least one child with clinical influenza in whom a rise in titer of antibodies for at least two of the strains of type B influenza virus was demonstrated. In none of these four was a significant rise in titer of antibodies demonstrated for any of the viruses used. The data on these four patients, however, are not so clear as in the group with the symptoms of colds. In one of them, Patient 29, there was only a single specimen of serum obtained eighteen days after the onset of symptoms and all of the titers were low. In each of two others, Patients 12 and 32, the initial blood was obtained ten days after the onset of fever and diarrhea. In the latter two the titers of antibodies for some of the type B

strains, particularly the titers obtained by the complement fixation test, were higher than in most of the acute phase sera of patients in whom a significant rise was later demonstrated. Finally, in the fourth, Patient 19, the symptoms began ten days after the initial specimen was obtained and only four days before the second sample of blood was drawn.

Household Contacts.—The results of the tests on the sera of the healthy household contacts of those patients in whom a rise in titer of influenza B antibodies was demonstrated are also of interest since little information is available concerning the influenza antibody response under just these conditions. There were twelve such household contacts in whom pairs of blood samples were obtained at the same time, as in the patients with clinical influenza. Four of the twelve were in Family F and two in Family N, and there was one in each of six other families. Unfortunately only two of these contacts were children. The rest were adults; almost all of them were parents of the patients, presumably being in fairly close contact with them. That was particularly true of mothers who in each instance nursed their children through the illness at home.

Half of these healthy contacts showed no rise in titer of antibodies against any of the four type B strains by either of the tests used. There were two individuals, Patients 2 and 5, both in Family F, in whose sera a fourfold rise in titer was elicited against the epidemic strain MB by the complement fixation test. In both of them the agglutinin inhibition test showed a twofold rise with the same virus, and similar slight rises which are not ordinarily considered to be significant also were noted in the complement fixation tests with the remaining type B strains. Four other contacts (Patients 3, 8, 11 and 17) each showed a twofold rise in titer of antibodies against one or more of the type B strains by either or both tests. There were only two instances (Patients 17 and 34) in which a twofold rise in titer was elicited with the PR8 strain by one or the other of the tests. Except for the elevated titers of agglutinin inhibition with PR8 mentioned earlier, the titers in the initial samples in all of these healthy contacts were essentially similar to those observed in the acute phase sera of the patients with clinical influenza in whom significant rises in influenza B antibodies were elicited.

Comparison of Agglutinin Inhibition and Complement Fixation Tests.—Except as already noted, the levels of the antibody titers obtained in any single specimen of serum by inhibition of hemagglutination and by complement fixation of the same virus usually corresponded fairly closely considering the limitations of the methods. The greatest and most frequent discrepancies were observed in the titers obtained with the PR8 strain: Several sera yielded high titers by inhibition of agglutination with this strain, but significantly high titers with the complement fixation tests were not observed. Except as noted earlier, the two tests also gave quite comparable titers in the same sera with each of the four strains of influenza B virus. In a few sera slightly higher titers with these viruses were obtained by complement fixation than by the inhibition of hemagglutination.

Tests With the BON Strain of Influenza B.—After all of these studies were completed, Dr. John F. Enders suggested that serologic tests with the BON strain also might be of interest. This strain was isolated by chick-embryo inoculation in Australia in 1943 by Beveridge and associates,[6] who showed it to be serologically related to, but readily distinguishable from, the Lee strain of influenza B. The results of the tests done on fifteen pairs of sera that were still available are shown in Table II.

TABLE II. RESULTS OF SEROLOGIC TESTS WITH STRAIN BON

	ANTIBODY TITERS			
	AGGLUTININ INHIBITION		COMPLEMENT FIXATION	
PATIENT	FIRST SPECIMEN	SECOND SPECIMEN	FIRST SPECIMEN	SECOND SPECIMEN
1	48	512	192Ac*	448
2	12	16	12	24Ac
4	8	6	16	10
7	8	160	12	576
8	6	8	5	6
9	6	6	10	5
10	12	512	10	768
11	16	16	32	20
12	16	12	40Ac	20
20	24	16	20	20
21	3	16	2	24
22	16	12	6	5
25	32	32	12	12
26	8	8	10	20
37	8	48	20	96

*Ac, Anticomplementary in lower dilutions.

Of particular interest is the demonstration of definite rises in antibody titers against the BON strain in Patient 21. In this respect the BON strain was similar to the WC and MF strains and unlike the Lee and MB strains. Tests were also done on the sera of six other patients with influenza including those from four (Patients 1, 7, 10, and 37) in whom definite rises in antibody titers had been elicited with the other type B strains and those from two others (Patients 25 and 26) in whom rises in antibody titers were not demonstrated. Similar results were obtained with the BON strain in each instance. The remaining eight persons whose sera were tested included two who were considered to have "colds" (Patients 9 and 22), one who had diarrhea (Patient 12), and five healthy contacts. The results obtained with the BON strain in the sera of these individuals also were essentially the same as those obtained with the other type B strains.

DISCUSSION

Among the present cases the serologic evidence clearly indicates that influenza B was the predominant if not the only virus implicated in the influenza outbreak at Needham. Evidence suggesting that probably only type B influenza occurred during the same period in Boston has also been obtained serologically and from the only successful isolation of virus from patients with acute influenza. Similar experiences were also reported from other localities at the same time.[7, 8] In a boys' school located several miles north of Boston, however,

an outbreak occurred during January, 1946, which was proved serologically to be due to influenza A, and no cases of influenza B infection were identified among students at that school.[9] Furthermore, among sporadic cases of clinical influenza which occurred in Boston a few weeks after the peak of epidemic prevalence of influenza B, several were identified serologically as influenza A. Included among the latter were persons who had previously suffered an illness, which was proved serologically to be influenza B during the epidemic, and were thus instances of consecutive infections with two distinct viruses.[5] Isolation of influenza A virus during December, 1945, was reported from Illinois,[10] and both influenza A and B infections were demonstrated in Australia earlier that year.[11]

The findings in Family W in which three seemingly typical cases of clinical influenza yielded negative serologic results have not been explained. These cases occurred during the height of the epidemic and one of them was in a pupil at the Junior High School. The cases may have been somewhat less severe than most of the others that were studied. Since a virus was not isolated from any of these cases, the possibility cannot be excluded that they had responded in a highly specific manner to their own strain but not to the related strains of influenza B virus used in this study. On the other hand, they may represent infections with a totally different virus or, what seems more likely, failures to respond with a rise in antibodies to typical strains, as Magill and Sugg[12] have shown in some cases of influenza A infection.

During the present outbreak in Needham, Mass., as well as in Boston and elsewhere, there were many patients with predominantly gastrointestinal symptoms interspersed among those with epidemic influenza. Some of these patients with so-called "intestinal flu" also had fever and other systemic symptoms such as malaise, headache, and prostration, which made it difficult to exclude the diagnosis of clinical influenza. The four cases included in this study and several others which were studied in Boston during the same time all failed to yield any definite serologic evidence of influenza by the development of antibodies to any of the viruses used in this study, in spite of close association of most of them with proved cases of influenza B.

Cases of common cold and other undifferentiated respiratory infections are always prevalent during the season when influenza outbreaks occur, and it is not always possible to distinguish isolated cases from those of influenza on purely clinical grounds. In the present study the four cases which clinically were considered to be common colds all failed to respond to their infection with a rise in antibodies to any of the viruses used, and the same was true of several additional cases studied in this laboratory during the same period. There were other cases, however, in which clinical differentiation was difficult and some of them proved serologically to be influenza B. The number of individuals of this type and of those with intestinal manifestations was too small to warrant any final deductions as to their relation to the prevalent influenza virus infections.

The same also may be said of the observations made on influenza antibodies in the healthy family contacts of patients with proved influenza B. The

present studies reveal no clear evidence of inapparent infection in these individuals, unless the slight rises in antibody titers can be interpreted in that way. Nor was any evidence found to indicate that the healthy contacts were protected by virtue of an adequate antibody content in their blood. These negative results may be a reflection of a type of resistance to infection among these contacts which could not be measured serologically with the available viruses or they may indicate an inapparent infection with an inadequate antibody response or with a strain of virus having low infectivity or low antigenicity in these individuals. Inapparent infections with influenza A as manifested by the development of antibodies in the absence of symptoms have been reported,[13-16] and influenza A virus has been isolated from apparently normal human contacts during an epidemic of influenza.[16] Serologic tests on contacts of patients with influenza B infections in an institutional outbreak in 1939 indicated an incidence of subclinical infection equal to that of clinical infections.[17]

Evidence was obtained which suggested antigenic differences between the recently isolated strains and the Lee strain of influenza B. Some cases which failed to show a rise to the latter reacted with a characteristic antibody response to some of the former. On the basis of the very meager evidence on Patient 21, together with the results on Patients 18 and 27, the strain MB from the Needham case appears to be antigenically similar to the Lee virus, whereas the strains WC and MF are more like the BON strain. There also was suggestive evidence of discrepancies between the antibody titers obtained by inhibition of hemagglutination and by complement fixation with the same viruses. The discrepancies were more frequent and more consistent (that is, the inhibition titers were always much higher) with the PR8 strain than with the B strains. With the latter viruses some titers were higher with one test and some with the other. These may be related in some way to the differences in antigenic components of the viruses as described by Wiener and associates.[18]

SUMMARY AND CONCLUSIONS

1. Serologic studies were carried out in cases and in family contacts during an outbreak of influenza which occurred in Needham, Mass., during mid-December, 1945.

2. The typical cases of influenza, with three exceptions, yielded evidence of infection with influenza B by a rise in titer of antibodies. The three exceptions were all individuals in the same family.

3. Some of the cases showed a rise in antibodies when tested with two strains of influenza B recently isolated from cases in Boston but failed to show such a rise in the same sera when tested with the Lee strain of influenza B and with a strain isolated from one of the cases in Needham.

4. Among the family contacts of the serologically proved cases of influenza B there were four who had symptoms which were more like those of the common cold (mostly coryza without fever) and four others who had low grade fever and predominantly gastrointestinal symptoms. No serologic evidence of influenza virus infection was obtained in any of these individuals.

5. Healthy family contacts of the proved cases of influenza B also failed to show significant rises in antibody titers in their sera for any of the strains of influenza B virus used or for the PR8 strain of influenza A, but slight rises were elicited in some of them.

6. There were some discrepancies between the titers obtained in the same sera by inhibition of hemagglutination and by complement fixation test with the same virus. These discrepancies were more frequent with the PR8 strain, and when they occurred, the agglutinin inhibition test with this virus gave the higher titers. Some sera gave higher titers in this test with the B strains, but more gave higher titers with the complement fixation test.

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THE EFFECT OF ANTIHISTAMINE DRUGS UPON SERUM-INDUCED MYOCARDITIS IN RABBITS

FRANKLIN A. KYSER, M.D., JOHN C. MCCARTER, M.D., AND JAMES STENGLE, M.D.
EVANSTON, ILL.

THE recent work of Rich and Gregory[7] upon the role of hypersensitivity in rheumatic fever once again has focused attention upon a problem that has perplexed investigators for many years. Before discussing the work to be presented in this paper, it would seem advisable to review briefly some of the pertinent contributions on this subject.

Swift[10] pointed out in a classic paper in which a complete review of the literature is given that the histologic picture of rheumatic fever may vary in different individuals, just as does the clinical picture, and that a patient who has had a rheumatic infection reacts more profoundly to the etiologic agent than does the individual who never has had rheumatic fever. He believes that this reaction is the result of a state of hypersensitivity. In a later communication Swift and co-workers[11] describe attempts to diminish the state of hypersensitiveness in rheumatic fever by giving repeated injections of a specific streptococcal vaccine. These workers believe that "if by this means of immunization the natural resistance of the tissues can be so enhanced that subsequent infection is met and tolerated with only a minimum of injury, the method will have been justified."

Gross and associates[4] attempted to produce rheumatic-like lesions by injecting various strains of streptococci isolated from patients with rheumatic fever into experimental animals. Their criteria for a diagnosis of experimental rheumatic fever were: (1) the Aschoff body, (2) nonbacterial pericarditis, and (3) nonbacterial verrucous endocarditis. These authors were unable to meet the criteria in their experimental lesions, and they stated that other workers had been too lax in their interpretation of results. The present paper also points out that perivascular accumulations occur in the normal rabbit heart.

Clark and Kaplan[2] presented a summary of the evidence that anaphylactic vascular lesions induced in animals are very similar to the histologic changes occurring spontaneously in rheumatic fever, periarteritis nodosa, and scarlet fever. The authors present the pathologic findings in two cases of serum sickness and suggest that the lesions are the result of a hypersensitive state. Rich[6] in a later paper again emphasized the role of anaphylactic hypersensitivity in patients who developed periarteritis nodosa following serum sickness.

In 1943 Rich and Gregory[8] published evidence that lesions resembling periarteritis nodosa can result from injections of sterile unpreserved horse serum into rabbits. In the same year work was presented in which lesions similar to rheumatic carditis were produced in rabbits by a similar method.

From the Evanston Hospital, and the Departments of Medicine and Pathology, Northwestern University Medical School
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Rich and Gregory[7] state that "the valvular and myocardial lesions which in their situation and basic characteristics conform so closely with those of rheumatic carditis that we believe they may provide a fair measure of support for the view that the specific rheumatic lesions may themselves be manifestations of an anaphylactic hypersensitive reaction." In this study eleven out of thirty-six rabbits developed rheumatic-like changes in the hearts. Rich and Gregory[7] describe in detail the characteristic cardiac lesions of rheumatic carditis and discuss ten major points as evidence that rheumatic lesions are hypersensitive in character. The histologic changes which were produced by Rich and Gregory were: (1) lesions similar to Aschoff bodies with the presence of Anitschkow myocytes or cells similar to them, (2) focal accumulations of mononuclear cells and large basophilic cells similar to those that characterize the Aschoff body, and (3) nonspecific lesions consisting of edema, round cells, and polymorphonuclear leucocytes.

Fox and Jones[3] repeated the work of Rich and Gregory and produced lesions similar to those of rheumatic carditis in twenty out of thirty rabbits. Robinson[9] was unable to produce cardiac lesions in ten rabbits injected with sterile horse serum; however, it must be noted that the animals in this work were killed in seven to twenty days, which probably accounts for the result obtained.

In 1945 Aikawa[1] summarized very completely the literature on this entire subject and discussed many papers which have not been mentioned in this presentation.

PRESENT STUDY

In the work herein reported the primary consideration has been to produce lesions in the rabbit heart according to the method of Rich and Gregory[8] and to note the effect of certain drugs upon the development of such lesions. No claim is made that the lesions are characteristic of rheumatic fever, although in many instances the changes produced bear a striking resemblance to the histologic picture of acute rheumatic carditis.

Thirty-nine young adult rabbits were used in our study. Of this group, nine animals died before the experiment was completed and the hearts of three of these nine were examined. However, our results are compiled only on those animals that lived throughout the entire study.

Sterile unpreserved horse serum was injected intravenously into rabbits according to the method of Rich and Gregory.[8] In one group of animals serum with preservative was used, and this fact will be emphasized in the results presented herein.

The animals were divided into the following groups:

Group I. Twelve rabbits received serum alone.

Group II. Nine rabbits received serum plus 2 mg. of beta dimethylaminoethyl benzhydryl ether hydrochloride (benadryl) two times daily subcutaneously throughout the duration of the experiment.

Group III. Six rabbits received serum plus 10 mg. of benadryl per kilogram of body weight two times daily subcutaneously throughout the duration of the experiment.

Group IV. Nine animals received serum plus an experimental drug* which has been shown to have antihistamine qualities, as measured by the Loew histamine spray test and by giving protection against anaphylaxis in guinea pigs. Throughout the remainder of this paper the drug will be named No. 1627. The dosage was 3 mg. per kilogram two times daily subcutaneously.

Group V. Three rabbits received serum alone and were used as controls for Group IV. In Group IV and V the third, or shocking, dose of serum contained a small amount of preservative, the significance of which is not known. For this reason the series of controls is kept in a separate group.

RESULTS

The results are summarized in Tables I to V.

Group I. Serum alone. Ten rabbits developed marked cardiac lesions. One rabbit developed moderate lesions. These consisted of the following types of changes:

1. Pericapillary and periarteriolar collections of lymphocytes and monocytes with occasional polymorphonuclear leucocytes. In many sections small areas of necrosis were found adjacent to blood vessels, surrounded with mononuclear cells, which resembled the Aschoff body. In many vessels the entire wall of the vessel was infiltrated with cells. Endothelial proliferation was marked, and in some sections hyalinization of the vessel wall was evident. Periarteriolar proliferations of fibroblasts frequently were present.
2. Scattered areas of hyalinization of muscle fibers.
3. Loose infiltrations with small round cells scattered between muscle fibers.
4. Occasional areas of vacuolar degeneration of fibers.

These changes are demonstrated in Figs. 1 to 6.

Group II. Serum plus benadryl. Four rabbits revealed no lesions or minimal changes. Two rabbits developed moderate cardiac lesions. In this

TABLE I. GROUP I SERUM ALONE

RABBIT	WEIGHT IN GRAMS	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
1	1,939	29	Marked
2	1,591	29	Marked
3	1,932	29	Marked
4	2,433	29	Marked
5	2,251	28	Marked
6	3,400	28	Animal died; not examined
7	3,747	27	Marked
642	2,000	29	Marked
643	2,000	29	Marked
644	2,500	29	Marked
645	2,450	29	Moderate
647	1,700	29	Marked

*Developed by G. D. Searle & Company, Chicago, Ill.

group the absence of perivascular changes was striking. Only in an occasional section were lesions found adjacent to blood vessels. No lesions resembling Aschoff bodies were seen. There were present, scattered between muscle fibers, small infiltrations of mononuclear cells and lymphocytes. Occasional areas of vacuolar degeneration were seen. Throughout all sections the lesions were very much less marked than those of Group I.

Fig. 1.

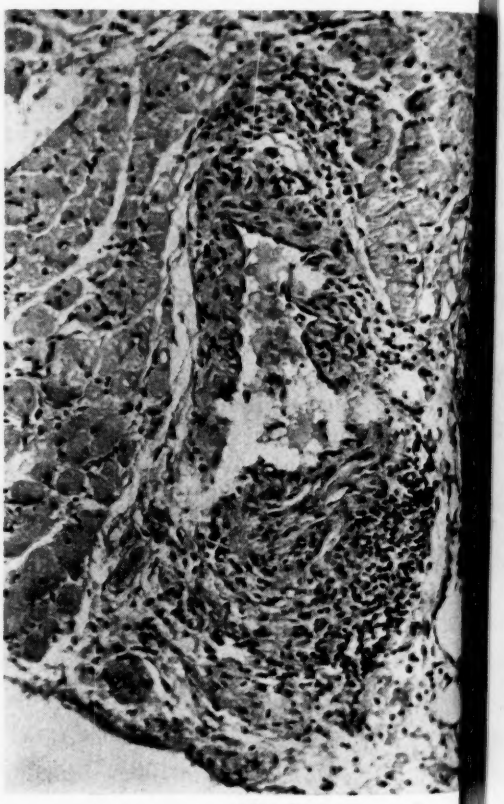

Fig. 2.

Fig. 1.—Arteriole with vacuolization of endothelium, hyperplasia, and swelling of media and surrounding dense infiltration of lymphocytes and polymorphonuclear leucocytes (×540).
Fig. 2.—Periarterial infiltration with lymphocytes and monocytes (×200).

Group III. Serum plus 10 mg. of benadryl per kilogram twice daily. Three rabbits showed no cardiac changes. Two animals revealed the presence of only an occasional perivascular infiltration, and these lesions were classified as minimal. One rabbit did develop a moderate number of lesions but not nearly as marked as in the control group.

Group IV. Serum plus drug No. 1627. Eight rabbits developed no lesions or only very slight changes. One rabbit developed moderate changes. Only occasional perivascular lesions were present. Nothing resembling an Aschoff body was seen. It must be pointed out that in this group and in Group V the third dose of serum contained a small amount of preservative.

TABLE II. GROUP II SERUM PLUS BENADRYL (2 MG. TWICE DAILY)

RABBIT	WEIGHT IN GRAMS	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
8	1,933	31	None
9	1,800	31	None
10	2,240	31	Moderate
11	1,962	4/28/46 to ?	Animal died; not examined
12	1,700	4/28/46 to ?	Animal died; not examined
13	2,005	4/28/46 to ?	Animal died; not examined
14	2,580	30	Very slight
15	2,430	30	Moderate
16	2,500	30	None

TABLE III. GROUP III SERUM PLUS BENADRYL (10 MG. PER KILOGRAM TWICE DAILY)

RABBIT	WEIGHT IN GRAMS	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
636	1,600	29	No lesion
637	2,400	29	No lesion
638	1,850	29	Moderate
639	1,600	29	No lesion
640	1,700	29	Minimal
641	2,200	29	Minimal

TABLE IV. GROUP IV SERUM PLUS DRUG NO. 1627

RABBIT	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
504	26	Very slight
505	26	Moderate
506	26	Very slight
507	26	Very slight
512	26	Very slight
513	26	None
515	9/ 3/46 to 9/20/46	Animal died; no lesions
576	9/ 3/46 to 9/20/46	Animal died; no lesions
577	26	Very slight

TABLE V. GROUP V SERUM ALONE

RABBIT	DURATION OF EXPERIMENT (DAYS)	RHEUMATOID LESIONS
578	26	Very slight
579	26	Marked
580	26	Marked

Group V. Serum alone to act as controls for Group IV. Two rabbits developed marked cardiac changes and one animal showed only slight changes.

DISCUSSION

From the literature reviewed and from the results herein reported, one is justified in stating that cardiac lesions may be produced in rabbits by the proper intravenous administration of horse serum. Gross and co-workers[4] state that perivascular infiltrations may occur in normal rabbits. In the present study

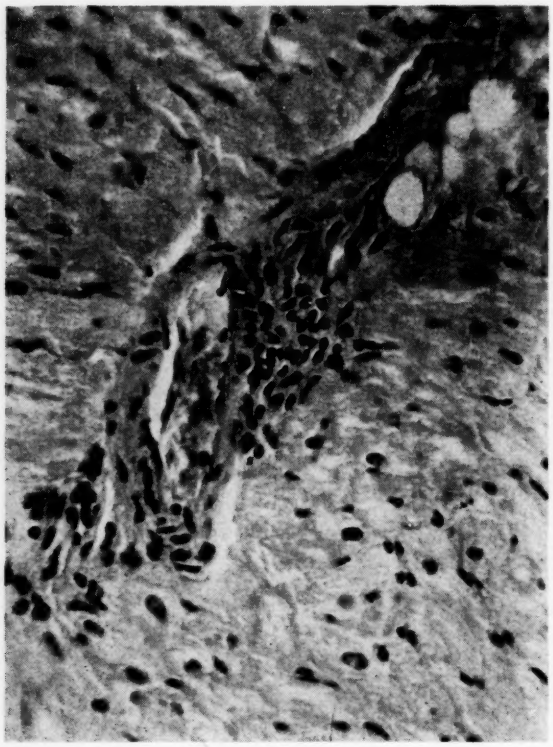

Fig. 3.

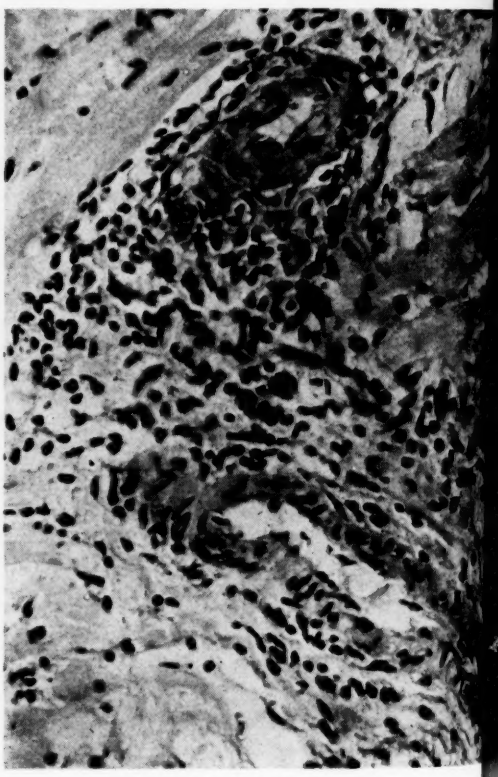

Fig. 4.

Fig. 3.—Lesion simulating an Aschoff body adjacent to arteriole (×450).
Fig. 4.—Lesion simulating an Aschoff body adjacent to arteriole (×540).

only a very occasional perivascular lesion was found in the animals that received benadryl and drug No. 1627. The control animals showed rather extensive lesions. We therefore feel that even if perivascular lesions do occasionally occur in the normal rabbit heart this fact does not alter the results of this experiment. Miller[5] has stated that 60 per cent of normal rabbit hearts will show cellular infiltrations between the muscle fibers. This may be true and, if so, will in no way change the evaluation of the results of this work, since most of our emphasis has been placed on the presence of vascular and perivascular lesions.

The similarity of the changes found in our control groups to the cardiac lesions of acute rheumatic carditis is striking. Since two antihistamine drugs have been shown to impede the development of these cardiac lesions in rabbits, it would seem advisable for further experiments to be carried out in an effort to determine the relationship of rheumatic fever to a hypersensitive state.

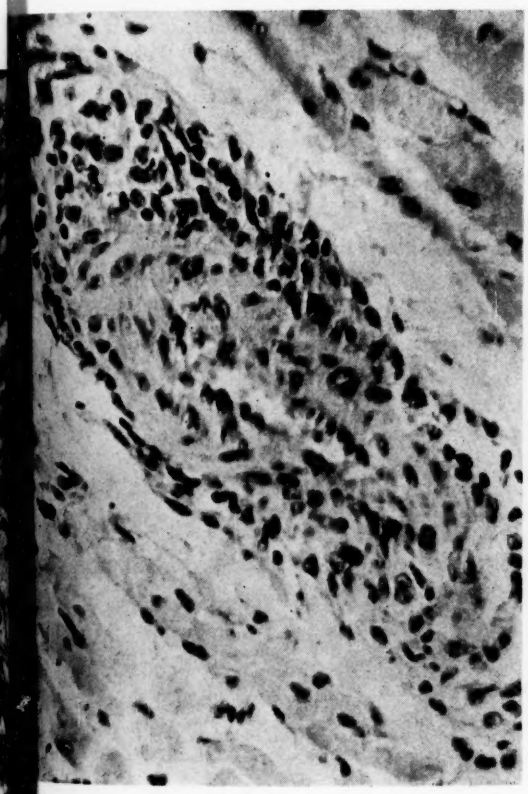

Fig. 5.

Fig. 6.

Fig. 5.—Infiltration around and into wall of artery by lymphocytes, monocytes, and eosinophiles; organizing thrombus is present in lumen of vessel (×540).

Fig. 6.—Periarteriolar infiltration (×500).

SUMMARY

Evidence is presented that benadryl and drug No. 1627, two antihistamine drugs, will impede the development of serum-induced myocardial lesions in rabbits. A brief review of the pertinent literature is included.

CONCLUSIONS

1. Myocardial lesions may be produced in rabbits by the injection of sterile horse serum according to the method of Rich and Gregory.

2. Benadryl and drug No. 1627 are effective in impeding the development of these lesions.

We wish to acknowledge the cooperation and help of G. D. Searle & Company, and of Dr. Irwin C. Winters of that company, for supplying drugs and animals for part of this work, and also Parke, Davis & Company, Detroit, Mich., for supplying the benadryl used in this investigation.

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EVIDENCE FOR THE ACTIVITY OF A SECOND MEMBER OF THE VITAMIN M GROUP (FERMENTATION FACTOR) IN SPRUE

A Case Report

Edgar Jones, M.D., Henry F. Warden, M.D., and
William J. Darby, M.D., Ph.D.
Nashville, Tenn.

INTRODUCTION

IN THE group of substances[1] loosely referred to as "folic acid" there are at least three related chemical compounds: (1) pteroylglutamic acid[2] (PGA), which has been synthesized and widely employed clinically and which is identical with the liver *Lactobacillus casei* factor[3]; (2) pteroyldiglutamylglutamic acid,[2] an *L. casei* factor which was isolated from "a fermentation residue," and, hence, was termed "fermentation factor,"[4] and which has not yet been available for widespread clinical testing; and (3) pteroylhexaglutamylglutamic acid[5] or vitamin B_c conjugate, isolated from yeast[6] and tested clinically in a limited number of patients.[7-9, 14] Our initial hypothesis that vitamin M deficiency in the monkey is the experimental analogue of sprue in man[10, 11] was based in part on the demonstrated activity of pure fermentation factor or pteroyldiglutamylglutamic acid in the vitamin M-deficient monkey and the demonstration of the activity of the related simpler compound, pteroylglutamic acid, in patients with sprue. It has since become evident that this simpler substance (PGA) possesses activity in the monkey.[12, 13] Suárez and co-workers[14] have reported a patient with sprue who was successfully treated with 4.9 mg. of pteroyltriglutamic acid daily intramuscularly. Castle and associates[15] reported that this material was inactive in patients with pernicious anemia, while Goldsmith[16] has found that it possessed some activity in a patient with nutritional macrocytic anemia. In a preliminary note, Spies[17] stated that a patient with pernicious anemia had exhibited a slight reticulocyte response following the administration of fermentation factor. We have had a sufficient quantity of isolated crystalline fermentation factor* to test its activity in one patient with sprue. Our experience with this case supplies additional evidence for the hemopoietic activity of the factor in this latter condition. Inasmuch as this isolated product is available in but limited quantities and our present supply is exhausted, it appears worth while to report this single case.

From the Departments of Medicine and Biochemistry, Vanderbilt University School of Medicine.

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*We are indebted to Dr. Y. SubbaRow and Dr. Stanton M. Hardy of the Lederle Laboratories, Inc., Pearl River, N. Y., for supplying us with this isolated crystalline fermentation factor, lot No. 7-6533.

CASE REPORT

A 52-year-old white woman entered Vanderbilt University Hospital Dec. 19, 1946, with the complaint of recurrent diarrhea of seven months' duration. There had been eight to twelve bowel movements daily for periods of several days to several weeks followed by intervals of four or five days free of diarrhea. The diarrhea was associated with lower abdominal cramping. The stools were described as watery to mushy, of increased bulk, yellow in color, and very foul in odor. Tarry stools had not been noted and gross blood had not been seen. There had been no jaundice, nausea, vomiting, or food intolerance. Moderate anorexia was present. Over the seven-month period there had occurred a weight loss of 80 pounds.

During the four months prior to admission the patient had noted progressive weakness, pallor, exertional dyspnea, and periodic soreness of the tongue. The weakness and dyspnea ultimately confined her to bed for the month preceding admission. There had been swelling of the lower extremities and the eyelids for three months and increasing grayish pigmentation of the hands, arms, and lower legs. No history of excessive blood loss was obtained. The patient's diet had consisted of milk, corn bread, turnip greens, and a few other vegetables; it had included very little lean meat.

Physical examination revealed an extremely ill woman lying flat in bed; she had marked pallor and pronounced generalized wasting of the subcutaneous and muscular tissues. She weighed 120 pounds (54.4 kilograms). The skin was loose, dry, scaly, and of a grayish appearance about the face, neck, arms, hands, and the lower portion of the legs. A few scattered petechiae were present. The hair was dry and without luster. The retinas revealed several small hemorrhagic areas both old and recent. The mucous membranes were pale. The mouth was edentulous. The filiform papillae were atrophic along the margins of the tongue. No abnormality of the lungs was detected. The heart was diffusely enlarged and a loud, low-pitched, blowing systolic murmur was audible over the entire precordium, with the point of maximum intensity in the fourth left interspace. The blood pressure was 98/60 and the pulse rate was 98 beats per minute. There was moderate thickening of the peripheral vessels. The abdomen was protuberant and some tenderness was present in both lower quadrants. The liver and spleen were not palpable and no abdominal masses were detected. Several small hemorrhoids were noted. Moderate pitting edema was present over the sacrum and the upper and lower extremities below the elbows and knees, respectively. The tendon reflexes were hyperactive. The ability to accomplish fine movements of the fingers was impaired and vibratory sense was diminished at the wrists and over the lower extremities. The plantar responses were flexor.

The data for the erythrocyte and leucocyte counts, reticulocytes, hemoglobin, and packed cell volume are given in Fig. 1. A differential count of the white blood cells showed the following percentages: segmented neutrophiles, 79; lymphocytes, 14; monocytes, 5; band forms, 1; and eosinophiles, 1. The platelets were scarce and the red blood cells showed marked anisocytosis and poikilocytosis. Aspirated sternal bone marrow revealed 6 per cent megaloblasts and 16 per cent erythroblasts, a picture compatible with a diagnosis of sprue or pernicious anemia. Gastric analysis revealed a histamine refractory achlorhydria. Other laboratory findings included: icteric index, 5; serum albumin, 2.58 Gm. per 100 c.c.; serum globulin, 2.16 Gm. per 100 c.c.; serum calcium, 7.9 mg. per 100 c.c.; and serum phosphorus, 2.4 mg. per 100 c.c. The stool fat content was 62 per cent of the dry weight. Repeated stool examinations revealed no occult blood, ova, or parasites, and no pathogenic organisms were found upon culture. The erythrocyte fragility did not differ from that of the control. The tourniquet test produced a few scattered petechiae. Prothrombin time was 41 per cent of normal. A cephalin flocculation test was negative after twenty-four hours and the thymol turbidity test showed 5 units. Serum levels of vitamins were as follows: vitamin C, 0.1 mg. per 100 c.c.; vitamin A, 39 I.U. per 100 c.c.; carotene, 9 to 16 μg per 100 c.c.; and tocopherol, 0.45 mg. per 100 cubic centimeters. The vitamin A tolerance test following the oral administration of 200,000 I.U. of vitamin A was as follows: three hours, 43 I.U. per 100 c.c.; five hours, 51 I.U. per 100 c.c.; and nine hours, 46 I.U. per 100 cubic centimeters. A

tocopherol tolerance[18] performed simultaneously with the vitamin A tolerance yielded a flat "curve" with values as follows: fasting, 0.45 mg.; three hours, 0.43 mg.; five hours, 0.38 mg.; and nine hours, 0.45 milligram. A glucose tolerance test was invalidated because of the accidental simultaneous subcutaneous administration of 1.5 mg. of histamine.

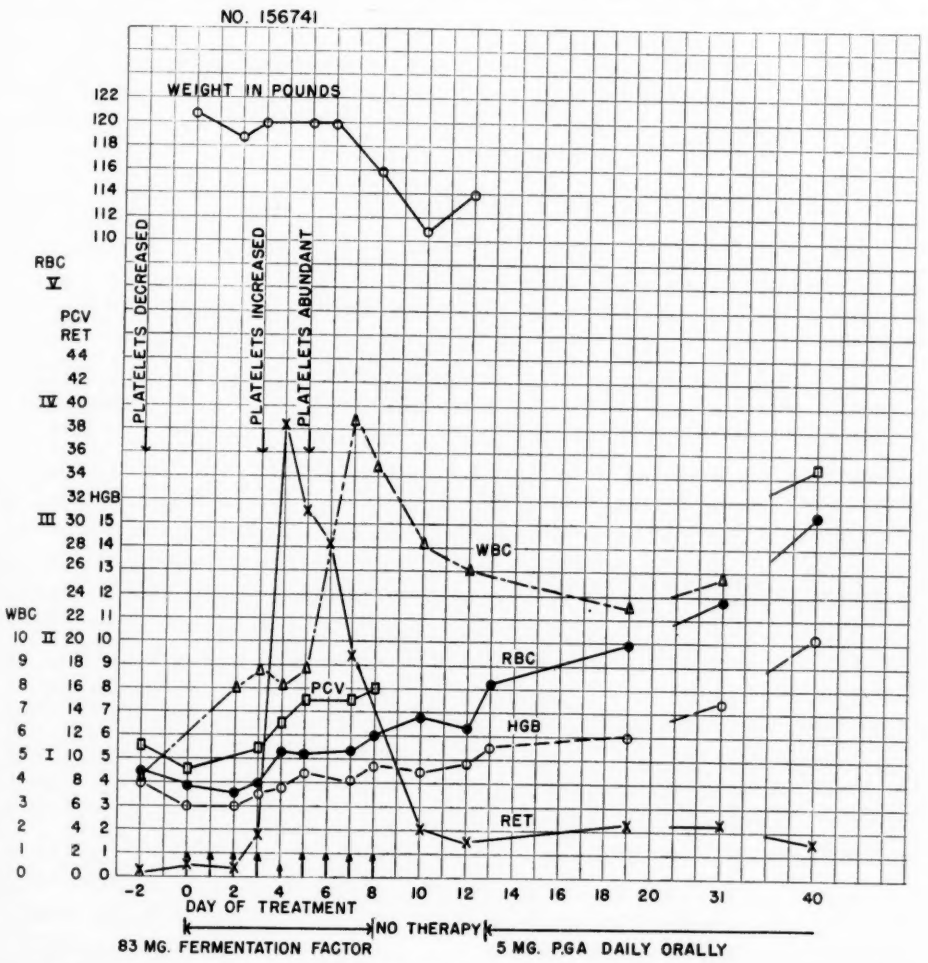

Fig. 1.—Hematologic response of patient with sprue following the intramuscular administration of fermentation factor. A total dose of 83 mg. was given during a nine-day period. Therapy was then discontinued for four days, following which it was resumed with 5 mg. daily of pteroylglutamic acid orally.

A diagnosis of sprue was made and on the second hospital day the patient was started on 5 mg. of fermentation factor intramuscularly twice daily. On the third day of therapy the reticulocyte count rose to 3.6 per cent, and on the fourth day a peak of 38.2 per cent was reached. The other hematologic data are presented in Fig. 1. On the second day of therapy the patient experienced a sense of well-being and considerable increase in appetite. On the fourth day beginning regeneration of the lingual papillae was noted. During the reticulocytosis the edema was pronounced, to subside within the next six days simultaneously with a decreased weight loss (Fig. 1). On the sixth day of therapy the stools were reduced in number to one or two per day. On the seventh hospital day the patient complained of pain in the left leg associated with tenderness over the femoral triangle. This was associated with

increased edema of this extremity, but there were no changes in skin temperature, color, or sweating. Homan's sign was negative. A lumbar sympathetic novocain block provided no relief. The pain and edema gradually subsided with elevation of the extremities.

A total quantity of 83 mg. of the fermentation factor was administered during the nine days. No additional therapy was instituted until on the thirteenth day of therapy when the patient left the hospital under protest because she wished to return home. She was discharged on 5 mg. of PGA daily by mouth. Continued evidence of hemoregeneration has occurred.

From the beginning of her hospital stay the patient exhibited fever; the temperature ranged from 100 to 100.6° Fahrenheit. Penicillin was administered in doses of 25,000 units every three hours for ten days at the end of which time the temperature became normal.

DISCUSSION

This case met the criteria which we have employed in the diagnosis of sprue.[10, 11, 19] The patient gave no history of having received previous therapy with liver, and she was maintained during the hospital stay on a diet free of organ meats. The sole hemopoietic agent administered was the pteroyldiglutamylglutamic acid. While spontaneous remissions can occur in sprue, the character and timing of the hematologic changes and the simultaneous clinical improvement indicate convincingly that this remission was due to the administered fermentation factor. It is not valid to attempt to make a quantitative comparison of the response of this patient to the fermentation factor with the expected or standard response of other patients, inasmuch as the course was complicated by thrombophlebitis which may have reduced the height of the maximum reticulocyte response and also the rate of regeneration of red cells. The response exhibited by our patient demonstrates that a patient with sprue who had not been treated previously with liver extract has responded to the fermentation factor, thus supplying the conclusive evidence called for by Suárez and associates[14] of the activity of this substance in sprue without "the participation of exogenous factors of liver extract." This finding, together with the similar clear-cut response of a patient with nutritional macrocytic anemia reported by Goldsmith,[16] indicates that this member of the vitamin M group is hemopoietically active for man as well as for monkeys.

The activity of crystalline fermentation factor in sprue is additional evidence in favor of the hypothesis[10, 11] that vitamin M deficiency in the monkey is the experimental analogue of sprue in man. Since both sprue and nutritional macrocytic anemia respond to fermentation factor, this is a further link in the evidence for the similarity of these two syndromes.

SUMMARY

A patient with sprue was treated intramuscularly twice daily with 5 mg. of fermentation factor (pteroyldiglutamylglutamic acid). A total quantity of 83 mg. was given over a period of nine days. A maximum reticulocytosis of 38 per cent occurred on the fourth day of therapy. This was accompanied by a rise in erythrocyte count and hemoglobin and clinical improvement.

These results furnish additional evidence of the activity of this member of the vitamin M group in man and of the correctness of the hypothesis that the experimental analogue of sprue is vitamin M deficiency in the monkey.

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THIOURACIL AND PROPYLTHIOURACIL: A COMPARATIVE CLINICAL STUDY

WILLIAM H. BEIERWALTES, M.D., AND CYRUS C. STURGIS, M.D.
ANN ARBOR, MICH.

EXPERIMENTALLY, Astwood and Vander Laan[1] found propylthiouracil to be ten times more effective than thiouracil by weight as a goitrogenic agent in rats. Because of this increased effectiveness, propylthiouracil has been used in smaller doses[2] than thiouracil to produce the same change in thyroid physiology without incurring such a high incidence of toxic reactions to the drug. On the other hand, we have shared with Bartels[3] the clinical opinion that propylthiouracil in the doses used at present is not as effective as thiouracil. Before increasing the dosage of propylthiouracil in our thyrotoxic patients, we decided to analyze our results to date statistically to prove or disprove this clinical opinion. In general, the results of this analysis presented herein do not indicate that an increase in propylthiouracil dosage over 150 mg. per day is advantageous in the treatment of thyrotoxicosis.

MATERIAL

Since March, 1946, we have used propylthiouracil as the initial form of treatment in forty-two thyrotoxic patients for a minimum period of six weeks. The distribution of patients treated with this drug, according to the number of months, in demonstrated in Table I.

Pertinent data necessary to compare the response rate of the two groups treated are presented in Table II. Other factors not listed in this table were identical in these two groups, since we directed the treatment in each group in the same manner but, starting in March, 1946, merely changed from thiouracil to propylthiouracil in the initial treatment of thyrotoxic patients. The general plan of treatment has been outlined in previous publications.[4-7]

Examination of Table II reveals major discrepancies at three points in these comparative data. In our opinion, the difference in the number of patients treated will not invalidate a comparison because (1) a statistical analysis of the first forty-seven patients treated with thiouracil[4] was not significantly different from the final analysis of the entire group of ninety-eight patients presented here and (2) we have continued the comparative graphs to be presented later in this paper only to the end of the first six weeks of treatment so that a maximum number of points would determine the position of the right end of the propylthiouracil line. The 25 per cent difference in the number of patients hospitalized

From the Department of Internal Medicine, University of Michigan, and the University Hospital.

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TABLE I. DISTRIBUTION OF PATIENTS TREATED WITH PROPYLTHIOURACIL BY NUMBER OF MONTHS

MONTHS	PATIENTS
1 to 2	12
2 to 4	15
4 to 6	12
6 to 8	3

TABLE II. FACTORS AFFECTING COMPARISON OF THIOURACIL- AND PROPYLTHIOURACIL-TREATED GROUPS

	THIOURACIL	PROPYLTHIOURACIL
Number of patients	98	42
Per cent hospitalized initially	76	50
Average period of hospitalization	5.5 wk.	5.4 wk.
Average initial B.M.R. per cent	38	41
Average frequency of B.M.R.s		
Inpatients	1 per wk.	1 per wk.
Outpatients	1 per 1.5 mo.	1 per 1 mo.
Per cent of nodular goiters	39	40
Average dose	0.6 Gm.	0.150 Gm.

initially in each group is insignificant in view of the graph comparing the results in each hospitalized group. The marked difference in drug dosage then is the only significant variable between the two groups.

RESULTS

The following graphs (Figs. 1 to 3) summarize the results of statistical comparison of the group of ninety-eight patients treated with 0.6 Gm. of thiouracil per day and the forty-two patients treated with 0.15 Gm. of propylthiouracil per day.

Illustrated in Fig. 1 is the fact that the rate of descent of the basal metabolic rate in both groups of hospitalized patients is almost identical. In general, the clinical change in the patient corresponded to the change in the basal metabolism.

It is demonstrated in Fig. 2 that in patients treated with propylthiouracil the initial response of the thyrotoxic state to treatment accompanied by bed rest is greater than when the patient is ambulatory, although the eventual result is apparently the same. Since all outpatients in our series were urged to spend a minimum of twelve hours a day in bed, there might be even greater discrepancies if the outpatients' activities were not restricted. It should be noted also that in general we have hospitalized the more severely toxic patients as an initial precaution.

An equal rate of improvement in the thiouracil- and propylthiouracil-treated groups is shown in Fig. 3. This similarity of response, in spite of a clinical opinion to the contrary, is reminiscent of our early use of thiouracil.[4] Although we did not feel that 0.4 Gm. per day of thiouracil was the optimum dose, a statistical comparison of the patients treated on 0.4 and 0.6 Gm. per day later revealed no significant difference in therapeutic effect. It should be noted that the basal metabolic rate in thyrotoxic patients in Michigan does not fall at the rate of 1 per cent per day, as noted by Bartels[8] for patients on the Atlantic Coast, but at a rate of less than 1 per cent every two days.

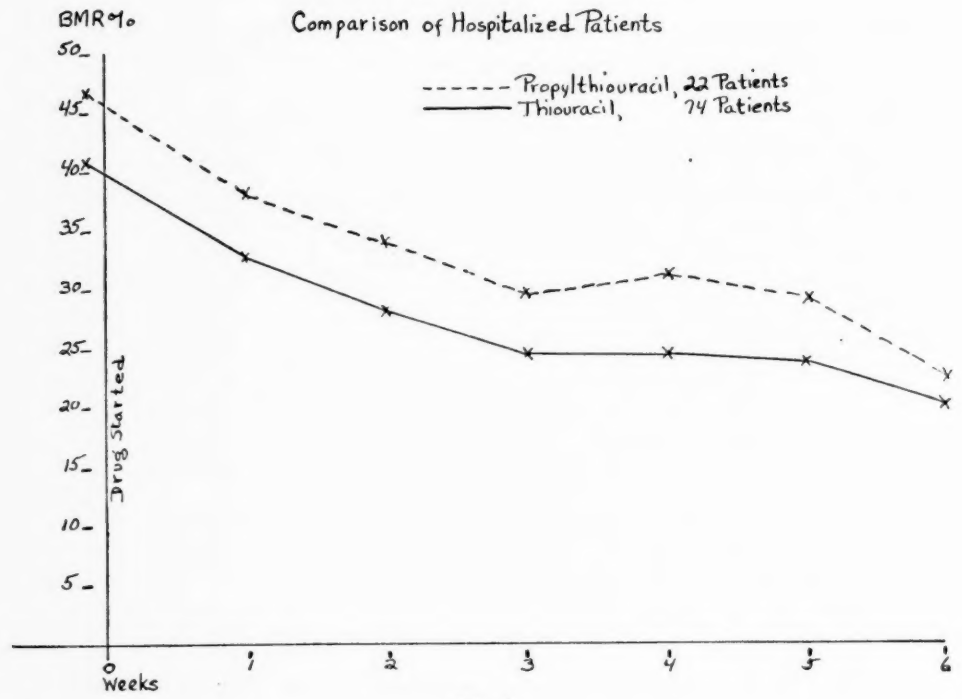
BMR%
Comparison of Hospitalized Patients
Propylthiouracil, 22 Patients
Thiouracil, 74 Patients
50
45
40
35
30
25
20
15
10
5
Drug Started
0
Weeks
1
2
3
4
5
6

Fig. 1.

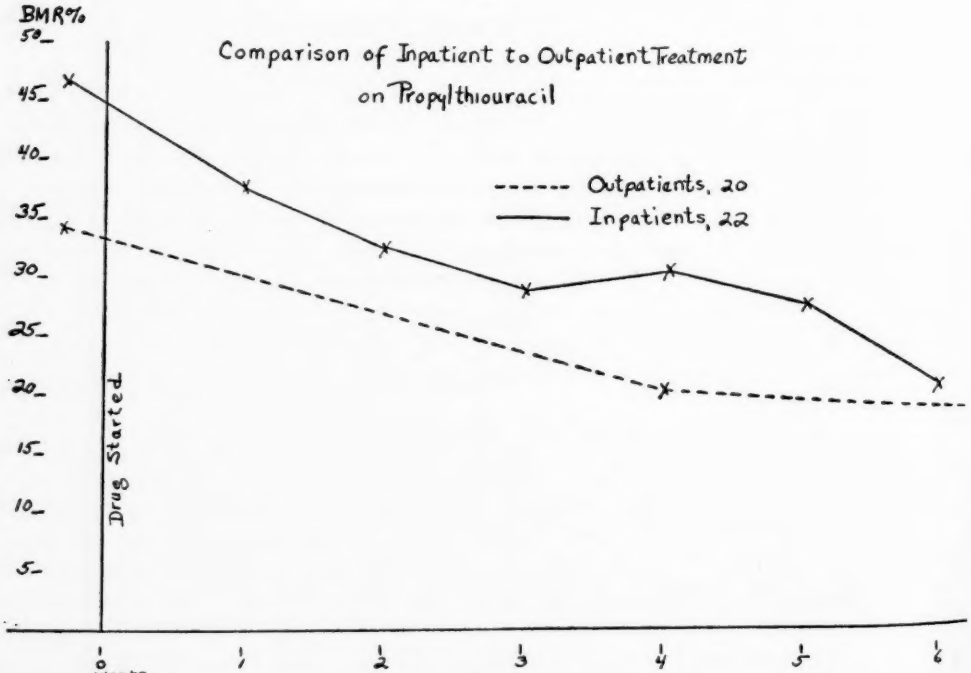
BMR%
Comparison of Inpatient to Outpatient Treatment
on Propylthiouracil
Outpatients, 20
Inpatients, 22
50
45
40
35
30
25
20
15
10
5
Drug Started
0
Weeks
1
2
3
4
5
6

Fig. 2.

DISCUSSION

This statistical comparison of the two drugs indicates that 150 mg. of propylthiouracil per day is an adequate dose for most thyrotoxic patients. The only toxic reactions we have observed in nine months of use of this new antithyroid agent are (1) drowsiness during the first two weeks of administration in about 30 per cent of the patients and (2) acneform dermatitis of the face in one patient, which disappeared when the dosage of the drug was reduced by 50 mg. per day. The lack of toxic reactions with this agent in our series is in keeping with the observation of Astwood and Vander Laan.[2] However, Bartels[3] mentioned three cases of agranulocytosis presumably due to propylthiouracil. Since propylthiouracil lacks toxicity mainly by virtue of its decreased dosage, the occurrence of the more severe forms of toxic reactions such as agranulocytosis and drug fever might be logically expected if the amount of drug administered is increased. Fortunately, our experience indicates that an increase in dosage is not necessary to achieve results comparable to those with thiouracil.

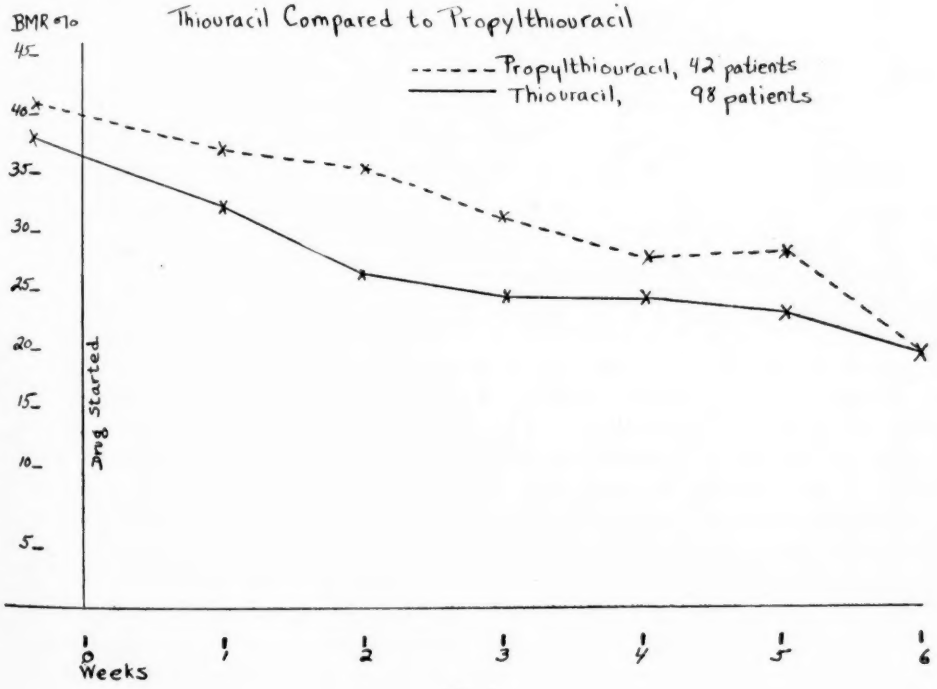

Fig. 3.

SUMMARY

1. A statistical comparison of ninety-eight thyrotoxic patients treated with 0.6 Gm. of thiouracil per day and forty-two treated with 0.15 Gm. of propylthiouracil per day reveals practically identical effectiveness of the two drugs in these doses.

2. An initial period of bed rest coupled with the administration of propylthiouracil is accompanied by a greater favorable initial response than in the ambulatory-treated patient, although the end result appears to be the same.

3. In Michigan the average rate of response of the basal metabolic rate of thyrotoxic patients treated with either drug is less than a 1 per cent fall every two days, in contrast to a fall twice as great reported from the Atlantic Coast.

4. The incidence of toxic reactions with propylthiouracil is much less than with thiouracil.

5. Severe toxic reactions well might be encountered if the present dosage of propylthiouracil is significantly increased.

6. The available data indicate that 150 mg. is probably the most satisfactory maximum dosage of propylthiouracil.

We are indebted to the staff of the Department of Internal Medicine and to Dr. F. A. Coller and Dr. R. W. Buxton of the Department of Surgery for their cooperation in making the patients available to us for study.

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OBSERVATIONS ON THE DIURNAL EXCRETION OF UROBILINOGEN IN THE URINE OF NORMAL SUBJECTS AND OF PATIENTS WITH LAËNNEC'S CIRRHOSIS

EDMUND PELLEGRINO, ARTHUR J. PATEK, JR., ANNE COLCHER, AND BEATRICE DOMANSKI
NEW YORK, N. Y.

THERE have been many reports on the diurnal variation of urobilin or urobilinogen excretion in normal and pathologic states. These studies have been performed with techniques differing in sensitivity and reliability and in the main on small groups of cases. In 1893 Grimm[1] and in 1897 Saillet[2] independently showed (1) that the excretion of urobilin during the day exhibits two peaks, one in midmorning and the other in midafternoon and (2) that nocturnal excretion of this substance constitutes a very small portion of the twenty-four hour excretion. Subsequent reports[3-10] are in general agreement that the maximal urobilin or urobilinogen excretion takes place in the afternoon and that minimal excretion takes place at night. Variations have been attributed to digestive and metabolic activity.

Wallace and Diamond,[10] for example, tested the urobilinogen excretion in morning and afternoon urines of 125 medical students. Their method depends upon the red color reaction produced by Ehrlich's aldehyde reagent with urobilinogen. A rough estimate of urobilinogen concentration is made by diluting the urine until the pink color disappears, a dilution of 1:20 being considered the outer limit of normal. Fifty specimens obtained in the afternoon showed a slightly higher concentration of urobilinogen than seventy-five specimens obtained in the morning. White and associates,[8] employing the Wallace and Diamond method, tested for urobilinogen at two-hour intervals during the day in fifteen normal subjects. "The variation in the results was found to be moderate and all within normal limits. . . . The afternoon specimens showed more urobilinogen than the morning specimens in about one-half of the cases." Steigmann and Dyniewicz[9] determined the hourly excretion of three normal subjects over a twenty-four hour period. In one subject the maximal excretion was at 4 P.M. and in one at 8 P.M., whereas the third subject showed peaks at 10 A.M. and 4 P.M. In comparing the morning and afternoon urine samples of twelve patients with urobilinogenuria from various diseases, Watson and coworkers[11] found the concentration of the afternoon specimens to be higher in nine of twelve instances.

Most of the preceding reports are concerned with the relative concentrations of urobilinogen excreted in morning and afternoon urine samples. It seemed desirable to measure the quantity excreted per unit time as well, since a highly concentrated urine might give a false impression of the total excretion. With

From the Research Service, First (Columbia) Division, Goldwater Memorial Hospital, Department of Hospitals, and the Department of Medicine, Columbia University.
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the introduction by Watson and co-workers[11] of a simplified method for the quantitative determination of urobilinogen, it became feasible to re-examine the problem of diurnal excretion of urobilinogen in a series of patients with liver disease. It was of practical interest to ascertain (1) whether there is an optimal period for urobilinogen excretion and (2) whether the daily pattern of excretion is constant in the same patient and in different patients. With this purpose the urobilinogen excretion was determined at three periods of the day in a series of normal subjects and in patients with cirrhosis of the liver.

METHOD

The method employed is essentially that described by Watson and associates[11] in which the Ehrlich color reaction is measured in a photoelectric colorimeter. In our procedure a few minor changes were introduced. Instead of a blank composed of reagents added in reverse order, as employed by these authors, we have employed a blank of 22 per cent HCl, which is the concentration of HCl contained in the Ehrlich reagent itself. The procedure is as follows: unknown, 2.5 c.c. urine, 2.5 c.c. "Ehrlich Reagent,"* 5 c.c. saturated sodium acetate; blank, 2.5 c.c. urine, 2.5 c.c. 22 per cent HCl,† 5 c.c. saturated sodium acetate.

It seemed essential to standardize the procedure with reference to time and temperature, since these factors influence the intensity of color reaction. The first two reagents are mixed and placed in a water bath at 37° C. for ten minutes. Colorimetry then is performed directly after the addition of the sodium acetate solution. The color obtained in this manner is generally maximal for the reaction.

Readings were made with the Klett photoelectric colorimeter with a No. 56 filter‡ and calibrated against the Pontacyl violet stock solution described by Watson and associates.[11]

Results are expressed in terms of Ehrlich units per hour (as proposed by Watson), since the reaction is not specific for urobilinogen but measures other Ehrlich-reacting substances as well. These chromogens are said to parallel roughly the excretion of urobilinogen.

The subjects of this study were thirty-six hospitalized patients with cirrhosis of the liver observed during a period of two years on the Research Service (First Division) of the Goldwater Memorial Hospital. The diagnosis was established in each case by history, physical findings, and laboratory tests of liver function. All but four cases had the diagnosis confirmed either by biopsy of the liver or by necropsy. Twenty cases were classified as in severe failure and sixteen in moderate failure. All patients were being treated according to a dietary regimen previously described.[12] This consisted of a nutritious diet, rich in protein and supplemented with 50 Gm. of brewers' yeast daily. No fluid or dietary restrictions were imposed preceding or during the test.

The patients voided at 7 A.M. Urine excreted from 7 to 10 A.M., 10 A.M. to 1 P.M., and 1 to 4 P.M. was then collected in separate bottles. Determinations were performed on each three-hour specimen within fifteen minutes of its collection. In this manner 180 series of determinations or a total of 540 single determinations were performed on thirty-six patients. The distribution of tests in this group was as follows: thirteen patients had one serial determination; five had three or four determinations; ten had five or more; and eight had ten or more.

The control series consisted of thirty-nine healthy subjects who were medical students and hospital personnel ranging in age from 20 to 35 years. Fifty-seven tridaily determinations or a total of 171 single determinations were made. The distribution of tests in this group was as follows: twenty-seven subjects had one serial determination; seven had two determinations; four had three; and one subject had four.

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*0.7 Gm. *p*-dimethylaminobenzaldehyde, 150 c.c. concentrated HCl, 100 c.c. distilled H_2O.

†150 c.c. concentrated HCl, 100 c.c. distilled H_2O.

‡There is little difference between values obtained with a No. 54 filter and those with a No. 56 filter. The procedure also has been carried out with a Junior Coleman colorimeter at 565 millimicrons.

RESULTS

The periods of maximal urine urobilinogen excretion both in normal subjects and in patients with liver cirrhosis are shown in Table I. There were fifty-seven serial determinations in normal subjects, of which sixteen were highest in the morning, nineteen at midday, nineteen in the afternoon, and three showed no peak of excretion. In patients with cirrhosis (180 serial tests), sixty-four showed maximal excretion in the morning, fifty-two at midday, and sixty-four in the afternoon.

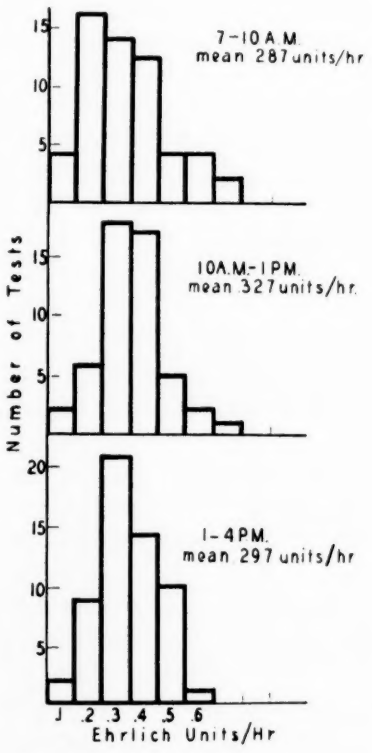

Fig. 1.

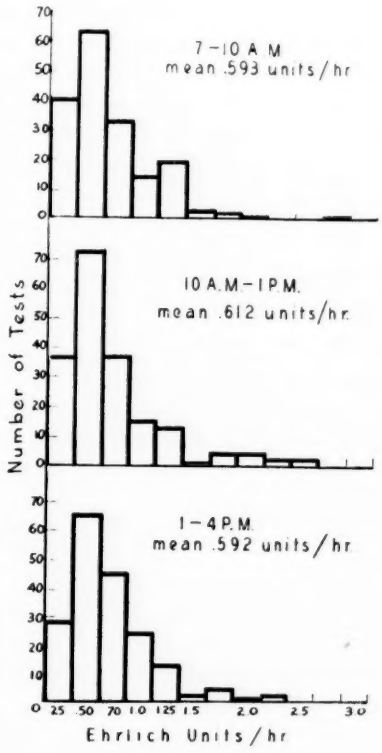

Fig. 2.

Fig. 1.—Diurnal urine urobilinogen excretion in 39 normal subjects. Mean values are similar for the three periods of the day. Few determinations exceed 0.5 unit per hour.

Fig. 2.—Diurnal urine urobilinogen excretion in 36 patients with liver cirrhosis. The pattern is similar to that of the normal subjects. Because of a wider distribution of values the scale has been reduced in this illustration.

The data are presented graphically in Figs. 1 and 2. Each histogram shows the number of determinations falling within a given range of urobilinogen excretion in units per hour. The distribution of these values does not differ significantly during the three periods, either for the normal subjects or the patient group. The similarity in the form of the graphs for the three periods suggests that no peculiar trend of urobilinogen excretion takes place during the day.

TABLE I. PERIODS OF MAXIMAL UROBILINOGEN EXCRETION

TIME	NORMAL SUBJECTS		PATIENTS WITH LIVER CIRRHOSIS	
	NUMBER OF INSTANCES	PER CENT	NUMBER OF INSTANCES	PER CENT
7 to 10 A.M.	16	29.6	64	35.5
10 A.M. to 1 P.M.	19	35.2	52	29.0
1 to 4 P.M.	19	35.2	64	35.5

The mean value of the normal subjects was 0.3 unit per hour. In 95 per cent of the tests made on normal subjects, values of 0.5 unit or less per hour were obtained. This finding is in agreement with the normal range obtained by Watson and co-workers.[11] In the case of liver cirrhosis the mean urinary excretion was 0.6 unit per hour. There was a much wider spread of values in this patient group, that is, from 0.03 to 5.4 units per hour. *Although all patients with cirrhosis of the liver showed increased urobilinogen excretion at one or another time, there were many tests that fell within the normal range.* In fact, fifty-five per cent of 540 tests on these patients fell within the normal range. The need for repeated tests becomes obvious.

The diurnal urobilinogen excretion of eight patients who had ten or more serial determinations made is shown in Table II. In each case the diagnosis of liver cirrhosis was confirmed by biopsy. Two points are illustrated in Table II: first, that while some patients favor one period for maximal excretion others do not; second, that the pattern of urobilinogen excretion may vary in the same patient at different times.

In general, there was a poor correlation between the severity of liver disease and the degree of urobilinogen excretion (Table II). The poor correlation stands out in contrast to the experience of others with acute hepatitis in which there appears to be close agreement between urobilinogen excretion and the activity of the disease process. However, when jaundice was present in patients with cirrhosis consistently higher values were obtained. This has been observed previously by Steigmann and Dyniewicz.[13]

Morning and afternoon peaks of excretion were noted in only a few instances. The peaks described by Grimm,[1] Saillet,[2] and Bang[5] were transitory and took place two or three hours after meals. Since the present study involves consecutive three-hour periods, those sharp variations might have been neutralized by the long periods of collection.

Salen,[4] Bang,[5] and White and associates[8] found no relation between the volume of urine and the amount of urobilinogen excreted. Our data tend to confirm their findings. However, it appears that urobilinogen excretion can be increased by excessive intake of water. This effect was tested in two normal subjects. After ingestion of 1,500 c.c. of water one subject showed little, if any, increase in urobilinogen excretion on two tests. In another subject the urobilinogen excretion after the ingestion of 1,500 and 5,000 c.c., respectively, was two and three times greater than that of the preceding control periods.

TABLE II. SUCCESSIVE TESTS OF URINE UROBILINOGEN EXCRETION IN EIGHT PATIENTS WITH LIVER CIRRHOSIS

PATIENT	CLINICAL SEVERITY	TIME	UNITS PER HOUR															
K.	4+	7 to 10 A.M.	1.02	1.44	1.10	5.10	1.20	1.13	.41	.24	.65	.88	.83	.32	1.17			
		10 A.M. to 1 P.M.	.54	.62	1.01	1.10	1.15	.71	.94	.34	.31	1.23	1.00	.92	1.70			
		1 to 4 P.M.	.51	.59	1.02	1.15	1.71	.79	.54	.98	.92	.48	1.24	.32	1.96			
M. D.	2+	7 to 10 A.M.	.48	.24	.26	.27	.51	.21	.40	.13	.73	1.24	.22	.46				
		10 A.M. to 1 P.M.	.28	.20	.63	.28	.27	.20	.28	.32	.41	.27	.35	.45				
		1 to 4 P.M.	.27	.22	.26	.35	.35	.15	.27	.19	.44	.32	.28	.56				
E. F.	4+	7 to 10 A.M.	.16	.16	.39	.41	.22	.24	.25	.29	.13	.03	.13	.09				
		10 A.M. to 1 P.M.	.05	.13	.58	.73	.42	.62	.24	.32	.33	.05	.18	.05				
		1 to 4 P.M.	.13	.09	.66	.25	.63	.50	.60	.44	.31	.06	.26	.19				
T. S.	1+	7 to 10 A.M.	.54	.80	.42	.73	.64	.89	.81	.39	.57	.75	.60	.43	1.13			
		10 A.M. to 1 P.M.	.43	.69	.45	.52	.32	.40	.79	.38	.57	.68	.54	.96	1.12			
		1 to 4 P.M.	.57	.68	.83	.99	.75	.68	.63	.58	.60	.55	.66	.59	.76			
H.	4+	7 to 10 A.M.	.52	.28	.39	.42	1.25	.53	.13	.28	.70	1.16						
		10 A.M. to 1 P.M.	.49	.29	.38	.57	.69	.30	.38	.27	.19	.53						
		1 to 4 P.M.	.32	.56	.60	.33	.31	.45	.27	.26	.35	.88						
C. G.	4+	7 to 10 A.M.	.67	.81	.32	.60	.79	2.63	.15	.25	.11	1.10	.80	.92	1.51	1.04	.61	.89
		10 A.M. to 1 P.M.	.31	1.05	.44	.43	.88	1.43	.64	.05	1.15	1.68	1.21	1.82	2.20	.51	2.51	.90
		1 to 4 P.M.	.46	1.07	.30	.35	.61	1.63	1.27	.21	1.59	1.74	.75	2.34	1.10	.45	.65	1.13
W. M.	2+	7 to 10 A.M.	.44	.37	.66	.41	.61	.43	.53	.85	.18	.33	.61	1.42				
		10 A.M. to 1 P.M.	.47	.36	.46	.48	.64	.60	.61	.49	.13	.25	.70	.89				
		1 to 4 P.M.	.41	.08	.64	.54	.52	.65	.23	.45	.41	.32	.52	.87				
M. D.	3+	7 to 10 A.M.	.75	.52	.54	.47	.50	.31	.50	.30	1.05	.66	.29	.55	.22	.47		
		10 A.M. to 1 P.M.	.40	.48	.31	.35	.48	.30	.49	.41	.30	.69	.25	.60	.34	.48		
		1 to 4 P.M.	.93	.19	.40	.24	.62	.53	.33	.43	.32	.25	.26	.53	.29	.30		

SUMMARY AND CONCLUSIONS

1. Urine urobilinogen excretion was determined for three successive periods of three hours each in thirty-nine normal subjects and in thirty-six patients with cirrhosis of the liver.

2. The mean urine urobilinogen excretion per hour was 0.3 Ehrlich unit for the normal subjects and 0.6 for the patients.

3. The period of maximal urobilinogen excretion varied in the different subjects and also in the same subject at different times.

4. There was a poor correlation between the degree of urobilinogenuria and the clinical severity of the liver disease.

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A COMPARISON OF THE NUTRITIVE VALUE OF THE PROTEINS IN MIXED DIETS FOR DOGS, RATS, AND HUMAN BEINGS

D. M. Hegsted, Virginia Kent, A. G. Tsongas, and F. J. Stare
Boston, Mass.

THE evaluation of the nutritive value of proteins has usually been done with rats, although some data have been collected using other species such as the canine, man, and the avian. In general, it is known that the supplementation of vegetable diets with a high quality protein such as meat causes an improvement in the nutritive value of the ration for all species which have been studied. The essential amino acids for the rat, human being, dog, and chick are similar but not the same. It seems likely that the quantitative relationships of the essential amino acids may also vary among the species. Such differences, should they occur, could only mean that various proteins would have different biologic values for different species. The relatively poor nutritive value of casein for the chick due to its low arginine and glycine content is a specific example, but whether such differences occur among the other common species of laboratory animals is unknown.

Biologic value, usually defined as the per cent of absorbed nitrogen retained in the body, depends upon the amino acid composition of the absorbed protein. There may also be species differences in the digestibility of proteins. Although the reasons for differences in the digestibility of proteins are obscure, specific amino acid linkages, proteinase inhibitors, amount of fiber in the food, method of preparation of the food or protein, and amount and kind of the various proteinases in the digestive apparatus are factors which may be involved. There is little reason to believe that all factors would be of equal importance in different species. A predominantly carnivorous animal with a short digestive tract might be expected to digest the proteins in vegetable matter less effectively than predominantly vegetarian species.

With these theoretic possibilities in mind, several diets which had previously been studied with human subjects[1] were fed to rats and dogs. In this paper the figures obtained for the digestibility and biologic values in these species are compared to those previously reported for man.

EXPERIMENTAL

The diets which were examined as protein sources have been previously described.[1] The all-vegetable diet called Diet IB contained no animal protein. Approximately 50 per cent of the protein in this diet came from white bread made without milk solids; other percentages were: 7.6 from corn meal, 4.4 from rice, 13.3 from potatoes, 16.3 from other vegetables (including lettuce, carrots, onions, and tomatoes), and 8.4 from fruits (including orange juice, apple sauce, and peaches).

From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School.
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When supplements were added, each constituent of Diet IB was decreased by one-third and the amount of protein thus removed was replaced by an equivalent amount of protein from the supplement. In this paper the diets are identified according to the supplement used, that is, meat, soy flour, wheat germ, or bread.

During the studies on human subjects[1] additional meals were prepared and dried at 40° C. in front of fans. After drying, the mixed diets were ground, analyzed for protein by the Kjeldahl method and for fat by ether extraction, and stored in the refrigerator until used in the present studies.

Studies With Dogs: Ten dogs were used in these studies. They varied in weight from 8 to 15 kilograms and were free from parasites. At the beginning of each study they were fed a diet essentially free of nitrogen of the following percentage composition: sucrose, 83; cottonseed oil, 9; salt mixture,[2] 4; and cod-liver oil, 2. Crystalline vitamins* were added to supply 200 γ thiamine chloride, 400 γ riboflavin, 200 γ pyridoxine, 1.5 mg. pantothenic acid, 2.5 mg. nicotinic acid, and 100 mg. choline per 100 Gm. of diet. The diet was fed in amounts to supply approximately 80 calories per kilogram of body weight. When the protein supplements were fed, the amount of the nitrogen-free diet was decreased proportionately to keep the caloric intake approximately constant.

In general, the plan followed was to feed the nitrogen-free diet for a period of ten to twenty days. The urine and feces were collected during the last week. The urine was combined to give composite samples representing a three- and a four-day period. The feces were homogenized in a Waring blender with water and combined in a similar manner. Charcoal markers were used to delimit the feces into appropriate periods. Following the nitrogen-free period, supplements of the experimental diets were given. Generally supplements of the all-vegetable Diet IB were fed for one week followed by a week during which the diet containing meat was used to supply a similar amount of nitrogen. During the next week another level of Diet IB was fed and following this the meat diet at a similar level, etc. However, in some animals only Diet IB was fed, the levels being increased each week. The amount of supplement which was given varied from 0.50 to 1.80 Gm. of nitrogen per dog per day.

Considerable difficulty was met in completing satisfactory studies. The diets were apparently not palatable for dogs, at least after a depletion period, and many of the dogs went off feed within a few days after the supplement was given and had to be discontinued. Thus, more data were collected on the all-vegetable Diet IB than on the diet containing meat, and studies on wheat germ and soy flour supplemented diets were not attempted with dogs. Studies similar to those reported by Allison and Anderson[3] were unsuccessful for similar reasons, since the higher levels of the diets were even more difficult to feed.

Studies With Rats: These studies were made with adult male rats. They were fed a nitrogen-free diet of the following percentage composition: sucrose, 84; corn oil, 10; salts, 4; cod-liver oil, 2; and vitamins at the same levels

*Supplied by Merck & Company, Inc., Rahway, N. J.

used in the diets for dogs. When supplements were added, these replaced sucrose and sufficient corn oil to maintain the fat content at 10 per cent. All supplemented diets contained 7.5 per cent protein (N × 6.25).

The experimental procedure was to feed the animals a nitrogen-free diet for twenty days, collections being made during the last eleven days in three periods of four, three, and four days each, respectively. Following this the diet containing protein from the all-vegetable diet was fed for twelve days, collections being made the last eleven days in four, three, and four day periods, respectively, as before. Then a similar twelve-day period followed during which one of the other diets was tested. The food intake during the nitrogen-free period was recorded and a like amount fed during the other periods. By this procedure each supplemented diet was compared to the all-vegetable diet. Twelve rats were used for each experiment.

Biologic values and digestibilities were calculated in the conventional manner. Digestibility is the per cent of fed protein absorbed and biologic value the per cent of absorbed nitrogen retained.

Studies also were made using young growing male rats (average weight 45 grams). In one experiment groups of six rats each were fed ad libitum on diets containing 7.5 per cent protein of a similar composition to those used for adult rats. In a second experiment using the same number of animals, each six animals of comparable weight receiving the different diets were restricted in food intake to the amount eaten by the animal having the lowest intake. This was usually the animal receiving the unsupplemented diet, but in some instances the bread-supplemented diet produced the least gain and food intake. In this study all the diets contained 9.6 per cent protein.

RESULTS

The results obtained with dogs are shown in Table I. The average results indicate an increase in digestibility and of biologic value of approximately 10 per cent by the inclusion of meat in the diet. The standard deviations are large for each value, especially of those for biologic value. It is our impression that there is a great variation in the ability of dogs to "handle" diets such as those which are high in vegetable matter.

TABLE I. DIGESTIBILITY AND BIOLOGIC VALUE OF DIETS FOR ADULT DOGS

SUPPLEMENT TO DIET IB	DIGESTIBILITY		BIOLOGIC VALUE	
	NUMBER OF TESTS	AVERAGE ± STANDARD DEVIATION	NUMBER OF TESTS	AVERAGE ± STANDARD DEVIATION
None	27	80.0 ± 7.7	16	67 ± 21
Meat	11	90.3 ± 7.1	11	77 ± 14

The data from the adult rats are shown in Table II. These data were treated somewhat differently than those from the dogs, since for each rat data were available on the all-vegetable diet for comparison with the supplemented diet. The values obtained for Diet IB in each experiment involving twelve rats are recorded, and the significance of the difference between this value and the observed value for the supplemented diet is given. It may be noticed

TABLE II. DIGESTIBILITY AND BIOLOGIC VALUE FOR ADULT RATS OF SUPPLEMENTED DIETS COMPARED TO ALL-VEGETABLE DIET

SUPPLEMENT	DIGESTIBILITY				BIOLOGIC VALUE				NET VALUE		
	DIET IB	SUPPLEMENT	DIFFERENCE	PROBABILITY OF SIGNIFICANCE	DIET IB	SUPPLEMENT	DIFFERENCE	PROBABILITY OF SIGNIFICANCE	DIET IB	SUPPLEMENT	DIFFERENCE
Meat	75.9	79.7	+ 3.8	0.15	68.1	80.7	+12.6	0.01	51.6	64.0	+12.4
Soy flour	79.1	91.4	+12.3	0.01	72.7	77.0	+ 4.3	0.10	57.4	70.2	+12.8
Wheat germ	83.3	89.2	+ 5.9	0.05	73.2	77.7	+ 4.5	0.03	61.0	69.1	+ 9.1
Average	79.4				71.3						

that whereas all three supplements allowed some improvement in both biologic value and digestibility, the increase in digestibility due to the addition of meat and the increase in biologic value from soy flour supplementation were not significant at the 5 per cent level.

The results with young growing rats are presented in Table III. In these studies all three supplements clearly improved the nutritive value of the diet, whether the diets were fed ad libitum or restricted to the intake of the poorest animal. In neither experiment are meat, wheat germ, or soy flour supplements significantly different. In these studies the diet containing additional bread studied with human beings[1] was also fed. A diet containing skim milk powder as the sole source of protein was included for comparison in this experiment. Skim milk powder appeared only slightly better than the meat-supplemented diet, while bread did not improve or decrease the nutritive value of the diet. The efficiency of protein utilization is not presented since, as has been shown,[4] this is a function of the weight of the animals.

TABLE III. WEIGHT GAIN IN YOUNG RATS RECEIVING VARIOUS DIETS

SUPPLEMENT TO DIET IB	AD LIBITUM FEEDING† (GM.)	RESTRICTED INTAKE‡ (GM.)
None	16.6 ± 7.0	8.0
Meat	61.0 ± 9.0	21.5
Wheat germ	57.0 ± 13.0	24.5
Soy flour	54.2 ± 16.3	22.5
Bread	17.5 ± 7.0	11.0
Skim milk powder*	73.0 ± 16.5	

*Skim milk powder as the sole source of protein, not as a supplement to Diet IB.
†During fifty days on diets containing 7.5 per cent protein.
‡During twenty-one days on diets containing 9.6 per cent protein.

In additional experiments groups of animals receiving the basal all-vegetable and the meat-supplemented diets were placed in activity cages. No significant differences in spontaneous activity were observed either with restricted or ad libitum feeding.

DISCUSSION

In the previous publication[1] the digestibility and biologic value of the protein in the various diets for human beings were estimated, although the excretions on a nitrogen-free diet were not actually determined. The fecal nitrogen on a nitrogen-free diet was estimated by feeding the all-vegetable diet at

three different levels and extending the regression line to zero protein intake. The urinary excretion on a nitrogen-free diet was estimated to be 2 mg. of nitrogen per basal calorie. The reliability and criticisms of these methods have been discussed,[5] but it is believed that they are useful for comparative purposes.

The present results have been summarized with those obtained on human subjects in Table IV. In general, it may be said that the results on rats and dogs are similar to those obtained with human beings although there are some apparent differences. Our most reliable data on all species were obtained with the all-vegetable diet, and it appears that digestibility of this diet was relatively better in man than in the other two species. The biologic values, on the other hand, are in the same range for all species, although somewhat lower for dogs. This may be some evidence for the correctness of the assumed figure for the endogenous nitrogen excretion used in the calculations on the data for man. The net protein values suggest that man utilized the all-vegetable diet somewhat better than the other two species, because of more efficient digestion. This result, however, may be due to the methods used in determining the metabolic fecal nitrogen. Bosshardt and Barnes[6] have shown that fecal nitrogen of mice receiving a nitrogen-free diet underestimates the metabolic fecal nitrogen occurring when proteins are fed. These authors believe that the method used in these studies on human beings is preferable to feeding nitrogen-free diets. If this is true, the digestibility of the proteins in rats and dogs has been underestimated. These studies appear to confirm the observations of Bosshardt and Barnes.

TABLE IV. COMPARISON OF NUTRITIVE VALUE OF PROTEINS OF VARIOUS DIETS IN MAN, RATS, AND DOGS

SUPPLEMENT	ADULT MAN			ADULT RATS			ADULT DOGS			YOUNG RATS GAIN IN THREE WEEKS	
	DIGESTIBILITY	BIOLOGIC VALUE	NET	DIGESTIBILITY	BIOLOGIC VALUE	NET	DIGESTIBILITY	BIOLOGIC VALUE	NET	AD LIBITUM (GM.)	LIMITED INTAKE (GM.)
None	87.5	72.5	63.5	79.4	71.3	56.5	80.0	66.7	53.0	16.6	8.0
Meat	89.0	80.4	71.5	79.7	80.7	64.0	90.3	76.7	69.0	61.0	21.5
Soy flour	83.8	70.6	59.2	91.4	77.0	70.2				57.0	22.5
Wheat germ	85.8	88.8	76.2	89.2	77.7	69.0				54.2	24.5
Bread	90.8	70.0	63.5							17.5	11.0

Man and the rat appeared to react in nearly identical fashion to the addition of meat to the diet. Digestibility was not improved, but the figures for biologic value were increased by approximately 10 per cent. The dogs, however, apparently digest the meat-supplemented diet better in addition to showing an increase in biologic value of 10 per cent. The overall net gain for the dogs was thus considerably more than in the other two species, 16 per cent compared to 8 per cent.

The data on man and the rats do not agree on the soy flour-supplemented diet. Whereas no improvement was noted by this addition for human beings in either biologic value or digestibility, both of these showed improvement in

the rats. Wheat germ supplements, while resulting in a gain in the net protein value of like amounts in the two species, showed the improvement in man entirely in biologic value whereas it was greatest in digestibility for the rat. As has been pointed out,[1] the data on soy flour and wheat germ in man are much less extensive than on the basal or meat-supplemented diet. Thus, we are unable to state that the apparent species differences in these two proteins are real.

The results on young growing rats are similar to those obtained with adult rats and, except for soy flour, also for those obtained with man. Supplements of meat, soy flour, or wheat germ apparently were of essentially equal effectiveness as they were in the adult animals. As in man, bread additions neither improved nor decreased the nutritive value. It is of interest that whereas meat supplementation caused an improvement of 8 per cent in the net protein value for adult animals, the relative improvement was much greater in the growing animals.

Recently Mitchell and Block[7] have presented convincing evidence that the biologic value of proteins as determined with rats bears a close relationship to their amino acid composition and may be estimated from the amino acid content. If, as suggested by the present data, the rat and man react similarly to various proteins, the value of the available data on proteins determined with rats is considerably enhanced. It further suggests that the percentage composition of the ideal protein for the two species is similar and lends support to the method of calculating amino acid requirements previously proposed by Stare, Hegsted, and McKibbin[8] and extended by Harte and Travers.[9] Since growing rats apparently classify proteins in a similar manner to adult rats, the results of such simple feeding experiments may be applicable for the evaluation of human dietaries as well.

CONCLUSION

Human subjects, rats, and dogs have been used to determine the biologic value of proteins in natural diets. The rat appeared to react in an exactly comparable manner to the human being when meat was added to the diet. Digestibility was not improved but the biologic value was increased 10 per cent. With the dog, on the other hand, both digestibility and biologic value were improved by meat supplementation.

The rat in these studies appeared more comparable to the human being than the dog.

Young growing rats, either by paired or ad libitum feeding, appear to classify these proteins in a similar manner to adult rats.

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PROTHROMBIN TIME STUDIES ON HUMAN BEINGS AND EXPERIMENTAL ANIMALS

G. A. Nitshe, Jr., H. W. Gerarde, and H. F. Deutsch
Madison, Wis.

THE one-stage assay of prothrombin has been shown to be affected by the concentration of fibrinogen.[1] By diluting a given plasma with a fibrinogen solution, sharper end points are realized at the higher dilutions, and the assay range may be extended to considerably higher plasma dilutions than is possible when the dilution of the substrate is not corrected. Preliminary in vitro prothrombin time studies had suggested that plasma fibrinogen concentrations might introduce changes in the apparent prothrombin levels as measured on saline diluted samples. The work reported here entails a study of the prothrombin time variable in experimental animals and in human beings as reflected by the clotting times of saline and fibrinogen dilutions of plasma in relation to the actual plasma fibrinogen level.

EXPERIMENTAL

All prothrombin determinations were carried out according to the method outlined by Deutsch and Gerarde.[1] The bovine fibrinogen was prepared according to the method of Seegers and associates.[2] It was used in the form of a 0.3 per cent solution (clottable fibrinogen) at pH 7.2 to 7.4. The major portion of the thromboplastin used in this work was prepared from rabbit brain according to the method of Quick.[3] One preparation however, was made according to the method of Link and associates.[4] After extraction of the thromboplastin with saline according to Quick's[3] method, an equal volume of a pH 7.4 buffer that was 0.05 M in cacodylate and diethyl barbiturate and 0.025 M in $CaCl_2$ was added. This mixture was centrifuged lightly and 1.0 c.c. portions of the supernatant were added to 0.5 c.c. of various saline or fibrinogen dilutions of a given plasma. The length of time for clotting at 25 degrees (± 1 degree) was determined visually. All plasmas were obtained by mixing 9 c.c. of blood with 1 c.c. of 0.1 M sodium oxalate, the resultant oxalated plasma being considered 100 per cent according to the usual convention. Fibrinogen assays were carried out by the method as outlined by Reiner,[5] the fibrin nitrogen being estimated by micro-Kjeldahl or colorimetrically following nesslerization.

Rats used for observations on the effect of caffeine, sodium salicylate, and vitamin K on prothrombin time were maintained two weeks or longer prior to the experiment on the relatively low vitamin K diet as used by Link and co-workers.[6] The rabbits used were fed exclusively on a commercial rabbit-pellet diet. All drugs with the exception of vitamin K were administered by stomach tube; the latter compound was given by intraperitoneal injection.

RESULTS

Caffeine Effects.—In the higher saline dilutions of plasma (10 per cent plasma) the small amount of fibrinogen present makes difficult the determination of the appearance of the relatively small fibrin clot formed. A plasma to which

From the Departments of Medicine and Physiological Chemistry, University of Wisconsin Medical School.

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various levels of fibrinogen have been added prior to saline dilution forms clots at 10 per cent plasma concentrations that are more readily discerned although no additional prothrombin has been added to the system.[1] It also has been indicated previously that the ingestion of caffeine by rabbits may alter the prothrombin time of saline dilutions of such plasma by virtue of its raising the fibrinogen level. In further testing this hypothesis rabbits were fed caffeine citrate at the level of 100 mg. per kilogram, and the prothrombin times of saline and fibrinogen dilutions of the plasma as well as the plasma fibrinogen levels were determined before and after such medication. Little variation in the prothrombin times of saline- and fibrinogen-diluted plasma was seen at the higher plasma concentrations (80 to 10 per cent). Therefore, prothrombin times were determined only for a 10 per cent dilution of plasma in 0.15 M NaCl and for 10 and 1 per cent dilutions in 0.3 per cent fibrinogen. Saline dilutions of plasma in excess of 1:10 (10 per cent plasma) were not carried out due to the difficulty and often impossibility of determining the end point of the clotting reaction. The results of these determinations on eight rabbits are shown in Table I. In addition, eight male rats weighing from 250 to 300 grams, which had been maintained on the relatively low vitamin K diet of Link and co-workers[6] for at least two weeks, were given 100 mg. of caffeine citrate per kilogram. The findings for these rats are shown in the caffeine data of (Table II). The marked increases in plasma fibrinogen values of these rats were found forty-eight hours after the ingestion of caffeine citrate. It can be seen from the data that the plasma fibrinogen levels vary considerably after treatment; in general, a distinct rise was noted which is in agreement with the work of Field and associates.[7] Although the 10 per cent dilutions of rabbit plasma in saline tend toward slightly shorter prothrombin times due to the enhanced fibrinogen levels, the effect is by no means clear-cut. Rat plasma showed slightly lengthened values in all dilutions as compared with the controls (Table II). The marked increases in plasma fibrinogen values of these rats were not reflected in shortened prothrombin times of the 10 per cent plasma in saline as would be expected from the differences of the saline and fibrinogen

TABLE I. EFFECT OF CAFFEINE ON FIBRINOGEN LEVELS AND PROTHROMBIN TIME OF RABBITS

RABBIT	FIBRINOGEN CHANGE IN MILLIGRAMS PER CENT*	PROTHROMBIN TIME (SECONDS)								
		10 PER CENT PLASMA IN 0.9 PER CENT SALINE			10 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN			1 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN		
		BEFORE	AFTER†	CHANGE	BEFORE	AFTER†	CHANGE	BEFORE	AFTER†	CHANGE
1	+168	23.8	21.9	−1.9	19.1	19.1	0	43.3	61.2	+17.9
2	+220	49.4	22.4	−27.0	18.9	17.1	−1.8	46.2	45.8	−0.4
3	+7	24.6	25.0	+0.4	20.4	21.1	+0.7	55.3	72.1	+16.8
4	+138	28.8	22.8	−6.0	20.6	21.0	+0.4	59.2	65.0	+5.8
5	+32	24.2	27.0	+2.8	20.2	20.9	+0.7	57.9	71.3	+13.4
6	−68	28.6	27.6	−1.0	20.1	21.0	+0.9	60.3	68.1	+7.8
7	+151	21.1	15.6	−4.6	16.5	15.6	−0.9	79.8	82.9	+3.1
8	+178	16.5	13.5	−3.0	13.4	13.4	0.0	45.7	60.7	+15.0

*Change in fibrinogen levels forty-eight hours after administration of 100 mg. caffeine citrate per kilogram.

†Prothrombin times forty-eight hours after the administration of 100 mg. caffeine citrate per kilogram.

TABLE II. EFFECT OF CAFFEINE, SODIUM SALICYLATE, AND VITAMIN K* ON FIBRINOGEN LEVELS AND PROTHROMBIN TIME OF RATS

GROUP	NUMBER IN GROUP	MILLIGRAMS PER CENT FIBRINOGEN (RANGE)	AVERAGE PROTHROMBIN TIME (SECONDS) 10 PER CENT PLASMA IN 0.9 PER CENT SALINE (RANGE)	10 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN (RANGE)	1 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN (RANGE)
Controls	20	215.0 (184 to 362)	34.5 (30.6 to 39.6)	23.9 (20.4 to 27.5)	80 (57 to 98)
	1†	0.0	>300	22.8	47
	1†	0.0	>120	20.4	69
Sodium salicylate	10	202.3 (90 to 328)	60.3 (42.7 to 90.2)	35.4 (23.2 to 51.8)	120 (65 to 195)
Sodium salicylate and vitamin K*	10	319.0 (115 to 520)	35.2 (31.3 to 39.9)	25.0 (22.3 to 28.2)	83 (55 to 107)
Vitamin K*	10	408.4 (298.0 to 497.0)	39.3 (37.7 to 42.0)	30.8 (27.9 to 33.4)	83 (57 to 104)
Caffeine	8	502.0 (420.0 to 640.0)	39.4 (32.8 to 51.8)	29.4 (25.5 to 41.3)	93 (66 to 186)

*The vitamin K used was Synkayvite (tetrasodium 2-methyl-1, 4-naphthohydroquinone diphosphoric ester).

†From a control group which showed no fibrinogen by the assay method used.

diluted systems of the 10 per cent rat plasma concentrations. The prothrombin times of the 1 per cent plasma in 0.3 per cent fibrinogen while indicating a mild hypoprothrombinemia do not fall in line with the results on rabbits (Table I), where even in the presence of a mild hypoprothrombinemia there were in general slightly shortened prothrombin times in the 10 per cent plasma concentration in saline of rabbits showing enhanced fibrinogen values.

As will be discussed later, there are various factors which may be tending to give results which are difficult to evaluate in the "one-stage" prothrombin assay. The interpretation by Field and associates[8] that shortening of prothrombin times as shown by some rabbits is due to an enhanced level of prothrombin is not borne out by the clotting times of 1 per cent plasma in fibrinogen. The lengthened prothrombin times of both rat and rabbit plasma at this dilution are indicative of a decreased amount of prothrombin as a result of caffeine ingestion. The results of the clotting times of 10 per cent rabbit plasma in saline are quite analogous to the results of Quick[9] who could demonstrate no hyperprothrombinemia as the result of caffeine feeding. Interesting is the report of Scherf and Schlachman[10] who found a rapid lowering of the prothrombin time of the plasma of patients who had received aminophyllin and theophyllin.

Salicylate and Vitamin K Effects.—It appeared that the hypoprothrombinemia experienced in rats as a result of salicylate ingestion[6] might likewise be a reflection of the assay technique as influenced by a lowered plasma fibrinogen level. Experiments studying the effect of sodium salicylate and vitamin K were carried out under conditions similar to those employed by Link and co-workers,[6] except for the inclusion of fibrinogen dilution prothrombin assay procedures.

These experiments were conducted on male rats weighing 250 to 300 grams and maintained under the same conditions as those on the caffeine experiment. The animals were given 100 mg. sodium salicylate by stomach tube and prothrombin studies were carried out approximately twenty hours later. The effect of such a level of sodium salicylate accompanied by an intraperitoneal injection of 5 mg. equivalents of vitamin K (Synkayvite: tetrasodium 2-methyl-l, 4-naphthohydroquinone diphosphoric ester), as well as the effect of vitamin K alone, was determined on individual groups of animals. The analytic findings resulting from this medication as compared with control rats receiving no treatment are shown in Table II.

It is apparent from the data that sodium salicylate induces a hypoprothrombinemia and that the effect observed by Link and associates[6] is not one of a decrease of fibrinogen level as suggested by Deutsch and Gerarde.[1]

Vitamin K counteracts the effect of the sodium salicylate; this effect does not appear to be due to the enhanced plasma fibrinogen level elicited by vitamin K administration. The data of Table II, however, in no way suggest that vitamin K can induce hyperprothrombinemia as has been stated by Field and Link[11] and Richards and Shapiro.[12] Mikhlin[13] has indicated that vitamins K_1 and K_2 as well as related synthetic products are without effect on the blood of normal animals and man. However, both Mikhlin[13, 14] and Babuk[15] report that a vitamin K concentrate prepared from maize stigmas accelerated the normal coagulability of the blood in the dog, rabbit, rat, and man. They attributed this effect to a hyperprothrombinemia, although in view of our findings it is possible that this noted effect may be due to other factors.

Afibrinogenemic Rats.—Interestingly in the control rats studied there appeared two afibrinogenemic animals. While 10 per cent saline dilution and even undiluted plasma failed to clot in the usual prothrombin assay, dilution of the plasma of these animals with fibrinogen gave prothrombin times which indicated normal or enhanced values of plasma prothrombin. It would appear that the diet used by Link[6] tends to produce low plasma fibrinogen levels. Similar responding animals have been found on this diet by Link.[16] These two animals showed no tendency toward hemorrhage as has been reported for afibrinogenemic human beings by various authors.[17-20] In fact, the animals studied here in whose plasma no fibrinogen could be demonstrated were able to survive heart puncture.

Thromboplastin.—In part, the discrepancy between the results of Field and associates[8] and of Quick[9] might be due to differences in the activities of the thromboplastin preparations used. We have found the prothrombin time of rabbit plasma at 25 degrees with the Quick[3] thromboplastin preparation to be shorter than that recorded by Link[4, 8] for rabbit plasma at 37 degrees. Although Quick[9] has likewise commented on the lowered activity of the Link[4] thromboplastin, we are not aware of a specific demonstration of such a difference. To test this experimentally, pooled macerated rabbit brains were divided into two equal portions and thromboplastin was prepared by both the method of Link[4] and that of Quick.[3] The prothrombin times of fibrinogen and saline

dilutions of rabbit and human plasma were determined using these thromboplastin preparations under identical conditions. The results for human plasma which were similar to those found for rabbit plasma are shown in Fig. 1 and clearly indicate the greater activity of the Quick[3] thromboplastin preparation. In this respect Scherf and Schlachman[10] used Russell viper venom as the thromboplastin which in comparison with the Quick thromboplastin is relatively low in activity. From this it appears that low activity thromboplastins may possibly show up plasma fibrinogen increases as apparent prothrombin increases. Prothrombin measurements of such plasma should be carried out at high plasma dilution in the presence of a constant substrate (fibrinogen) level if the results of the one-stage prothrombin time assay method are to be interpreted in terms of prothrombin content.

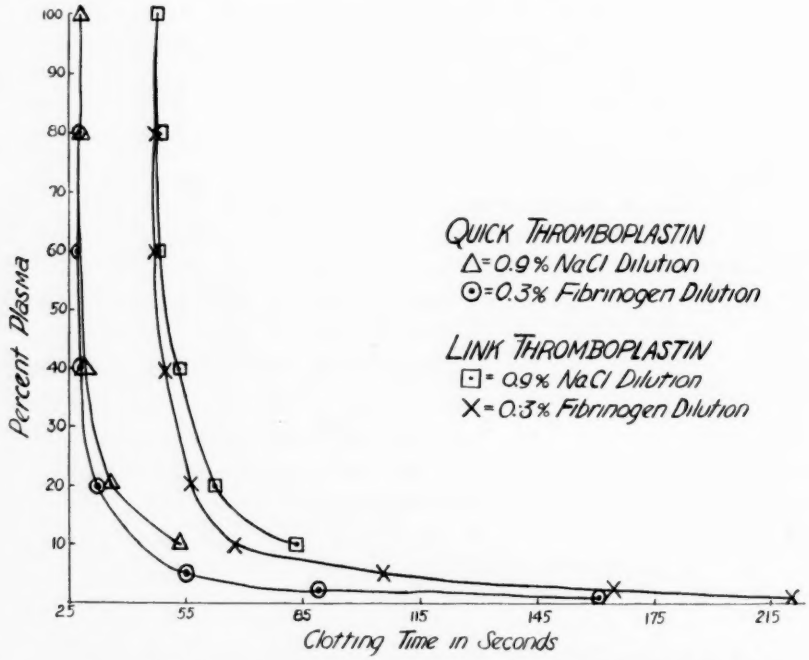

Fig. 1.—Comparison of clotting times of saline and fibrinogen dilutions of a single human plasma using Link[4] and Quick[3] thromboplastin.

Prothrombin Variations in Human Beings.—The relatively wide variations in prothrombin levels for essentially normal rats and rabbits as reflected by the clotting time of their 1 per cent solution of plasma in fibrinogen are interesting. It has been claimed by Quick[21] that the prothrombin levels of normal human beings fall within a narrow range. Other authors, however, indicate that considerable variations exists.[22, 23] Warner and associates[24] indicate that the effects of plasma antithrombin are minimized by high dilution of the plasma. Prothrombin assays at a plasma concentration of 1 per cent in the presence of a constant level of fibrinogen are probably more indicative of prothrombin content than assays

TABLE III. PROTHROMBIN TIME AND FIBRINOGEN LEVELS OF NORMAL HUMAN BEINGS ARRANGED ACCORDING TO INCREASING LEVELS OF FIBRINOGEN

PATIENT	MILLIGRAMS PER CENT FIBRINOGEN	PROTHROMBIN TIME (SECONDS)		
		10 PER CENT PLASMA IN 0.9 PER CENT SALINE	10 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN	1 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN
1	190	44.3	40.0	193
2	207	40.1	32.9	186
3	220	33.0	30.3	176
4	225	41.1	36.5	144
5	260	36.6	32.7	198
6	272	39.5	25.2	141
7	277	35.8	33.1	180
8	280	38.5	34.4	156
9	297	37.7	32.5	147
10	303	37.7	33.7	161
11	304	38.3	32.8	128
12	311	35.3	32.5	124
13	317	44.2	38.2	177
14	319	38.2	32.1	158
15	327	34.4	34.6	164
16	343	39.8	32.7	148
17	344	36.8	35.4	162
18	346	38.0	34.4	144
19	347	36.2	31.0	143
20	376	38.1	34.3	159
21	432	39.2	37.1	192
22	439	36.1	31.4	142
Average	306	38.1	33.5	160
Range	(190 to 439)	(34.4 to 44.3)	(25.2 to 40.0)	(124 to 198)

conducted in relatively concentrated plasma or in saline dilutions where the enzyme substrate tends to become the limiting factor. Application of the fibrinogen dilution technique was applied to the plasmas of a series of normal human subjects. Considerable differences in the prothrombin levels as reflected by variations in the prothrombin time were found by assay of 1 per cent plasmas. The variations encountered in the saline-diluted 10 per cent plasma were relatively smaller. These data are shown in Table III. There appears to be little correlation between plasma fibrinogen levels and the variations in the prothrombin times of plasma diluted to a level of 10 per cent with saline or fibrinogen. In general, however, the fibrinogen-diluted plasmas showed a slightly shortened prothrombin time.

To determine whether markedly enhanced plasma fibrinogen levels would affect the prothrombin time, a series of clinical cases tending in this direction were studied. They represented varied pathologic conditions with plasma fibrinogen values extending from normal to extremely high levels as shown in Table IV. The prothrombin times of 1 per cent plasma in fibrinogen were analogous to those found in normal individuals. However, the individual variations encountered were much greater. This was true also for the 10 per cent plasma clotting times. A noticeable difference in many cases was the lengthening of the prothrombin times of the 10 per cent plasmas diluted with fibrinogen as contrasted to the saline dilution. This was particularly evident in the samples of plasma having the higher fibrinogen levels. In the normal series,

TABLE IV. PROTHROMBIN TIMES AND FIBRINOGEN LEVELS OF HUMAN BEINGS WITH VARIOUS DISEASES ARRANGED ACCORDING TO INCREASING LEVELS OF FIBRINOGEN

PATIENT	DIAGNOSIS	MILLIGRAMS PER CENT FIBRINOGEN	PROTHROMBIN TIME (SECONDS)		
			10 PER CENT PLASMA IN 0.9 PER CENT SALINE	10 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN	1 PER CENT PLASMA IN 0.3 PER CENT FIBRINOGEN
1	Atrophic cirrhosis	288	60.2	55.8	277.0
2	Carcinoma, uterus; x-ray therapy	288	34.4	30.5	129
3	Tuberculosis	298	47.5	47.3	239
4	Carcinoma, uterus; x-ray therapy	309	36.8	32.5	140
5	Atrophic cirrhosis	310	41.4	38.6	203
6	Chronic ileitis	313	44.0	41.4	253
7	Infectious hepatitis	320	40.7	39.6	248
8	Carcinoma, colon	336	46.3	45.5	203
9	Anxiety tension state	354	40.2	37.5	149
10	Sinusitis and otitis media	392	39.6	36.4	133
11	Osteomyelitis	393	38.1	31.2	122
12	Lues with retinitis	393	37.3	35.4	205
13	Carcinoma, breast	403	35.2	33.4	154
14	Tuberculosis	410	43.0	44.2	106
15	Carcinoma, uterus; x-ray therapy	413	33.9	29.8	131
16	Furunculosis	416	39.8	34.1	96
17	Atrophic cirrhosis	435	47.2	44.6	215
18	Myelogenous leucemia	444	46.1	47.2	201
19	Carcinoma, larynx	470	36.9	35.3	169
20	Rheumatic fever	477	33.7	31.8	137
21	Multiple myeloma	489	36.3	34.2	96
22	Carcinoma, bladder	490	36.7	32.6	148
23	Tuberculosis	525	41.1	45.6	226
24	Tuberculosis	540	35.6	34.2	155
25	Carcinoma, uterus; x-ray therapy	549	29.2	27.6	107
26	Carcinoma, breast; x-ray therapy	560	30.9	30.0	130
27	Arteriosclerotic gangrene	586	33.2	30.9	133
28	Subdiaphragmatic abscess	589	33.3	33.6	146
29	Hypertensive heart disease	591	42.2	42.5	244
30	Carcinoma, esophagus	594	36.4	35.5	201
31	Carcinoma, uterus; x-ray therapy	610	37.4	38.0	132
32	Peritonitis	615	43.0	43.4	153
33	Cholecystitis with lithiasis	619	41.0	43.5	193
34	Carcinoma, stomach	637	34.4	35.8	139
35	Constrictive pericarditis	657	38.5	41.7	202
36	Epididymitis	672	44.0	45.7	172
37	Carcinoma, prostate	691	31.9	29.7	125
38	Multiple burns	754	32.3	33.3	138
39	Xanthochromatosis	777	37.0	38.5	161
40	Thrombophlebitis	778	33.0	31.3	116
41	Carcinoma, esophagus	820	34.4	36.4	141
42	Bursitis and tendonitis	828	35.7	33.2	125
43	Rheumatic fever	1,040	31.7	33.4	142
	Average	522.6	38.4	37.2	164
	Range	(288 to 1,040)	(29.2 to 60.2)	(27.6 to 55.8)	(96 to 277)

as previously noted in all except one instance, the prothrombin times of 10 per cent plasma diluted with fibrinogen were shorter than the corresponding saline dilutions.

In previous in vitro studies fibrinogen was added to plasma to provide a system containing variable amounts of fibrinogen with a constant level of prothrombin.[1] Dilution of such a plasma with saline shows that the fibrinogen addition definitely affects the apparent prothrombin level as indicated by shorter prothrombin times in the higher dilutions. In our present work similar high fibrinogen level plasmas as occurring in various pathologic conditions were studied. The results (Table IV) are difficult to appraise. It would appear that fibrinogen fluctuations might be accompanied by variation in such clotting entities as inhibitors and prothrombin. A possible factor tending to introduce variations is the rate at which prothrombin is converted to thrombin. Thus, the discrepancies of prothrombin content of various animal plasmas as determined by the one-stage method of Quick[21] and the two-stage method of Warner and associates[25] appear to be conversion rate differences rather than absolute prothrombin levels. It is possible that even within a given species this conversion rate may vary. In addition, the presence of inhibitors for prothrombin, thrombin, and thromboplastin likewise affect clotting reactions. The true evaluation of the blood clotting mechanism as revealed by "one-stage" prothrombin assays should take into account all of these factors as well as the factors we have studied. Since simultaneous control of all recognized clotting factors in the "one-stage" type of assay would be extremely difficult, it would appear that assay techniques built around the "two-stage" assay would be more feasible for the true evaluation of prothrombin. Here individual reactions can be somewhat segregated, controlled, and studied independently. The "one-stage" clotting picture represents the summation of various reactions, and it is difficult to appraise the effect of any one given reaction.

SUMMARY

Various experimental and clinical conditions involving variation in fibrinogen and prothrombin levels have been studied.

The apparent hyperprothrombinemia experienced by some workers as a result of caffeine feeding appeared as a mild hypoprothrombinemia when prothrombin time assays were carried out at a 1 per cent plasma concentration in the presence of 0.3 per cent fibrinogen. The effect of sodium salicylate on rats is to induce a definite hypoprothrombinemia with little alteration in fibrinogen level. Vitamin K while counteracting the action of sodium salicylate *does not* in itself produce hyperprothrombinemia. A series of normal human subjects and clinical patients showed relatively wide variations in prothrombin levels as revealed by prothrombin times of 1 per cent dilutions of plasma. The marked fluctuation in plasma fibrinogen content did not correlate with expected prothrombin times of saline dilutions; this indicates a need for the evaluation of clotting factors in addition to those studied here.

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THE CORPUSCULAR CONSTANTS OF COLLEGE WOMEN OF THE NORTH CENTRAL STATES*

GLADYS KINSMAN LEWIS,† MARGARET A. OHLSON,‡ DENA CEDERQUIST,§ AND EVA G. DONELSON||

HEMATOLOGIC data on 4,550 normal college women, 16 to 30 years old, inclusive, were reported recently by Ohlson and associates.[1] Included in these were data from 1,348 individuals, 17 to 24 years old, inclusive, on whom the hemoglobin concentration, the number of red cells, and the packed cell volume were determined on the same samples of blood. From these values, which include data from the nutrition laboratories of Iowa State College, Kansas State College, University of Minnesota, and Oklahoma Agricultural and Mechanical College, the corpuscular constants of Wintrobe[2] have been calculated. Complete information on the experimental methods used in the study were given in the previously mentioned report.[1]

The corpuscular constants (mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration) were first introduced and defined by Wintrobe[2] in 1929. In 1934 he[3] summarized the data available at that time and reported values for the corpuscular constants on approximately 700 individuals, 274 of whom were normal women between the ages of 18 and 30 years, inclusive, from different parts of the United States and Denmark. The mean values for the women of this age range were: mean corpuscular hemoglobin, 28.8 micromicrograms; mean corpuscular volume, 87.0 cubic microns; and mean corpuscular hemoglobin concentration, 33.1 per cent. Since 1934 the corpuscular constants have been determined for subjects of both sexes in different parts of the world.[4-14] In 1942, while referring to values for corpuscular constants found since 1934, Wintrobe[15] stated, "Reports made since that time, with one exception, are either in exact agreement with those given or differ so slightly that the mean values remain the same when these additional data are used."

The present study of the corpuscular constants of 1,348 college women offers the largest series of values reported to date.

RESULTS AND DISCUSSION

In Table I are given the number of subjects and the mean values for mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration for each group of women studied and for the subjects as a

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‡Present position: Head of Department of Foods and Nutrition, School of Home Economics, Michigan State College, East Lansing, Mich. With the assistance of Virginia Minnich and Gertrude Hoppe Brash. Journal paper No. J-1369 of the Iowa Agricultural Experiment Station, Ames, Iowa, Project 538.

§Contribution 133, Department of Home Economics, Kansas Agricultural Experiment Station, Manhattan, Kansas.

||Paper No. 2283, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, Minnesota.

TABLE I. MEAN VALUES FOR MEAN CORPUSCULAR HEMOGLOBIN, MEAN CORPUSCULAR VOLUME, MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION, STANDARD DEVIATIONS OF MEANS (S), AND STANDARD ERRORS OF MEANS (S_x) OF COLLEGE WOMEN OF FOUR STATES, WITH APPENDED VALUES FOR THESE CORPUSCULAR CONSTANTS AS REPORTED BY WINTROBE FOR WOMEN OF SIMILAR AGE RANGE

STATE	SUBJECTS	M.C.H. MEAN (γγ)	M.C.H. S	M.C.H. S_x	M.C.V. MEAN μ^3	M.C.V. S	M.C.V. S_x	M.C.H.C. MEAN (%)	M.C.H.C. S	M.C.H.C. S_x
Iowa	593	28.8	3.0	0.12	89.5	4.2	0.17	32.2	2.4	0.10
Kansas	220	29.1	2.3	0.16	91.7	4.8	0.32	31.7	2.2	0.15
Minnesota	292	30.7	2.1	0.12	92.7	6.6	0.39	33.2	1.8	0.11
Oklahoma	243	29.2	2.4	0.15	88.3	5.2	0.33	33.1	2.3	0.15
All four states	1348	29.3			90.3			32.5		
Wintrobe's report on women from the United States and Denmark	274	28.8			87.0			33.1		

whole. The standard deviations and the standard errors of the means are also included for each state group of subjects. For ease of comparison the values for corpuscular constants of the women between the ages 18 and 30 years, inclusive, given in Wintrobe's 1934 report have been included in the table.

The mean values of the corpuscular constants are somewhat different for the four groups of women studied. The respective maximum and minimum mean values are: mean corpuscular hemoglobin, 30.7 and 28.8 micromicrograms in Minnesota and Iowa, respectively; mean corpuscular volume, 92.7 and 88.3 cubic microns in Minnesota and Oklahoma, respectively; and mean corpuscular hemoglobin concentration, 33.2 and 31.7 per cent in Minnesota and Kansas, respectively. It is interesting to observe that the Minnesota group, in which the bloods were taken uniformly under basal conditions, show the highest values for all three corpuscular constants, although the mean values for hemoglobin, number of red cells, and packed red cell volume were the lowest of any state group.[1] Only in one of the four states, Iowa, is the mean corpuscular hemoglobin as low as 28.8 micromicrograms, the mean value reported for women of a similar age range by Wintrobe. In no instance is the mean corpuscular volume as low as Wintrobe's 87.0 cubic microns. In two states, Minnesota and Oklahoma, the values for mean corpuscular hemoglobin concentration are practically the same as the 33.1 per cent reported by Wintrobe, while in the other two states lower values are found.

A study of the significance of the differences between the mean values for the four groups shows that significant differences occur for all three corpuscular constants (Table II). For mean corpuscular hemoglobin, three out of six possible comparisons show highly significant differences between means, that is, critical ratios of 3.0 or more. Highly significant differences are found in five out of six instances for mean corpuscular volume and in four out of six for mean corpuscular hemoglobin concentration.

Even though there are significant differences between the means for each constant, there seems to be ample justification for putting the data of the four state groups together to obtain mean values of the constants for the 1,348 subjects as a whole. The reasons that prompt such a conclusion are (1) the marked inter- and intraindividual variations observed in the different states during the

TABLE II. SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS FOUND IN RESPECTIVE STATE GROUPS FOR MEAN CORPUSCULAR HEMOGLOBIN, MEAN CORPUSCULAR VOLUME, AND MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION EXPRESSED AS CRITICAL RATIO*

		OKLAHOMA	IOWA	KANSAS
Mean corpuscular hemoglobin	Minnesota	7.8	10.6	8.0
	Oklahoma		2.4	0.5
	Iowa			1.5
Mean corpuscular volume	Minnesota	8.0	7.4	2.0
	Oklahoma		3.2	7.4
	Iowa			6.1
Mean corpuscular hemoglobin concentration	Minnesota	0.5	6.7	8.0
	Oklahoma		5.0	6.6
	Iowa			2.8

*C.R. $= \frac{m_1 - m_2}{S_{x\ Diff.}}$, where $S_{x\ Diff.} = \sqrt{\frac{S_1^2}{N_1} + \frac{S_2^2}{N_2}}$; m_1 and m_2, S_1 and S_2, and N_1 and N_2 denote the means, the standard deviations of the means, and the number of cases, respectively; and $S_{x\ Diff.}$ stands for the standard error of the difference between the means.

present study and (2) the wide variation in the data used by Wintrobe to establish his means for these constants. The range of mean values used by Wintrobe was: 28 to 31 micromicrograms for mean corpuscular hemoglobin, 82 to 93 cubic microns for mean corpuscular volume, and 32 to 34 per cent for mean corpuscular hemoglobin concentration.

By reference to Table I, it is seen that the corpuscular constants for the subjects of the present study as a whole may be compared readily with the comparable values reported by Wintrobe for women of a similar age range. The respective values are: mean corpuscular hemoglobin, 29.3 and 28.8 micromicrograms; mean corpuscular volume, 90.3 and 87.0 cubic microns; and mean corpuscular hemoglobin concentration, 32.5 and 33.1 per cent. The similarity of these values in each case is quite striking, particularly so for mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration. When one considers that Wintrobe's range for mean corpuscular volume, 82 to 93 cubic microns, was much wider than for the other constants, the difference of 3.0 cubic microns would seem to have little, if any, greater significance than the differences found for the other constants.

This study and a previous one,[1] as well as numerous other reports in the literature, serve to emphasize the fact that the mean values for hemoglobin, red cell count, and packed red cell volume, and the mean corpuscular constants derived therefrom, show considerable variation between the different groups studied in this country and abroad. The lack of uniformity in these values, even when the numbers of subjects in the studies are large, leads to speculation as to the reasons for these differences. To what extent they are due to the experimental procedure employed and what role environmental conditions (temperature, humidity, altitude), race, and dietary factors play in their origin are points that are not clearly understood and require further elucidation.

SUMMARY

1. Values for mean corpuscular hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin concentration are reported on 1,348 women from

colleges or universities in four states, Iowa, Kansas, Minnesota, and Oklahoma.

2. The mean values found for these corpuscular constants are somewhat different for the four state groups. The respective maximum and minimum values are: mean corpuscular hemoglobin, 30.7 and 28.8 micromicrograms; mean corpuscular volume, 92.7 and 88.3 cubic microns; and mean corpuscular hemoglobin concentration, 33.2 and 31.7 per cent.

3. When the results on the 1,348 subjects are considered as a whole, the mean values for the corpuscular constants are 29.3 micromicrograms for mean corpuscular hemoglobin, 90.3 cubic microns for mean corpuscular volume, and 32.5 per cent for mean corpuscular hemoglobin concentration.

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TWO POPULAR FALLACIES REGARDING Rh

Preliminary Report of Some Thought-Provoking Observations

First Lieutenant Paul G. Hattersley, Medical Corps, Army of the United States

WITH the tremendous mass of literature on the Rh factor which has accumulated in the seven years since its discovery, it is inevitable that certain fallacious ideas should have reached print and should persist in the minds of many of their readers. One or two of these fallacies have been repeated in one paper after another without an adequate investigation of their origin or of their accuracy.

It is the purpose of this paper to present a preliminary report of some observations made in this laboratory which would seem to indicate the fallaciousness of two commonly held beliefs regarding Rh.

Fallacy 1: Only a Small Percentage of Rh-Negative Persons Can Become Sensitized by Transfusion With Rh-Positive Blood.—Wiener[1-3] has stated variously that between one in fifty and one in twenty-five Rh-negative persons who are "exposed to the Rh antigen" become sensitized, and his statements have been repeated widely elsewhere. He further states[4] that the incidence of sensitization by Rh-positive pregnancy, and by transfusion, is about the same. Unger[5] goes so far as to say that "The fact is now recognized that when repeated transfusions of Rh-positive blood are given to an Rh-negative patient, antibody response to the Rh antigen develops in approximately one in twenty-five patients." Yet, neither Wiener nor Unger refers to experimental or clinical data as a basis for his statements.

Actually there is much recent evidence from several sources that sensitization may occur in many more than one in twenty-five Rh-negative individuals who receive Rh-positive blood. Diamond and co-workers,[6] in a series of 2,500 servicemen who had been transfused under wartime conditions without regard for Rh type, were able to demonstrate anti-Rh antibodies in the serum of about 50 per cent of the Rh-negative individuals. Ross,[7] in a smaller series, likewise found Rh antibodies in nearly 50 per cent of the Rh-negative bloods.

In a similar survey now under way in this laboratory, we have examined bloods of a group of servicemen who have at some time in the past received transfusions, presumably without the benefit of Rh typing. These bloods were classified as Rh-positive or Rh-negative, according to their reactions with standard Rh_0 typing serum. All the Rh negatives and the majority of the Rh positives were subtyped with sera of Rh′ and Rh″ specificity. All sera were examined then for Rh antibodies according to the method of Diamond and Denton,[8] a single drop of serum being incubated in a small tube with a drop of

From the Laboratory Service, Letterman General Hospital, San Francisco, Calif.

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2 per cent suspension of Rh_1Rh_2 cells in 30 per cent bovine albumen. Parallel tests were set up using Rh-negative cells. Sera agglutinating the Rh-positive cells were then titrated, using the same cell suspension both in saline and in albumen. The serum dilutions for the latter were made in normal serum, as suggested by Levine and Bernstein.[9] For comparison, all sera showing anti-Rh activity were also tested with the blocking technique of Wiener.

The results closely approximate those of Diamond and of Ross. Of the first 122 bloods examined, twenty were negative for the Rh_0 factor; of these, two were type Rh′ and one was Rh″. Of these twenty sera, eleven (or 55 per cent) contained Rh antibodies, all of the blocking or incomplete variety, and all were inactive against saline-suspended cells. Titers against Rh_1Rh_2 cells in albumen ranged from 1:1 to 1:5120, while blocking titers ranged from 0 to 1:32. The findings are summarized in Table I.

TABLE I. TITERS WITH Rh_1Rh_2 CELLS

SERUM	SALINE	ALBUMEN	BLOCKING
1	—	1:4	1:1
2	—	1:64	1:2
3	—	1:2	1:1
4	—	1:8	1:4
5	—	1:32	1:2
6	—	1:128	1:16
7	—	1:8	1:4
8	—	1:4	1:1
9	—	1:5120	1:32
10	—	1:64	1:4
11	—	1:1	—

It is evident that if these sera had been examined with saline-suspended cells only, all would have been discarded as containing no antibodies. Yet, many of these patients had had one or more transfusion reactions, a few quite severe. There can be little question that they were sensitized to the Rh factor by transfusion.

In evaluating these findings it should be kept in mind that in choosing the subjects for this survey, all patients were included who gave any history of receiving whole blood overseas, or at small hospitals in this country, regardless of the number of transfusions or the elapsed time since the last one. Most of them had not received blood for many months. It seems quite likely that if they had been examined sooner after their last transfusions, the percentage showing antibodies would have been even higher.

Further evidence of this sort comes from the work of Diamond[6] and of Wiener and Sonn-Gordon[16] in sensitizing Rh-negative volunteers by repeated injections of Rh-positive blood. Although there was a great deal of variation in the ease with which the volunteers were sensitized, the percentage who eventually developed antibodies when sensitizing doses were continued was extremely high.

It would appear then that we must discard the notion that only one in twenty-five Rh-negative persons who are transfused with Rh-positive blood may become sensitized. Rather, it must be assumed that every Rh-negative patient is capable of developing Rh antibodies and should be transfused only with Rh-

negative blood. Transfusion reactions due to Rh sensitization are probably much more common than often is realized. Most of them can and should be avoided.

Fallacy 2: Blocking Antibodies Are Always of Rh_0 Specificity.—In 1944 and 1945 Race,[10] Wiener,[11] and Diamond[12] independently reported the discovery of an antibody which, while it did not of itself agglutinate Rh-positive cells, specifically combined with them so as to prevent their agglutination by a known Rh agglutinating serum. This blocking antibody was described as specific for the Rh_0 (D) element of the cells, blocked Rh_1 cells behaving like Rh′ cells, and blocked Rh_2 cells like Rh″. This specificity has repeatedly been emphasized in the literature. Wiener[4] has even suggested that this phenomenon be used in identifying antisera of unknown specificity, blocked Rh_1 and Rh_2 cells being used in place of Rh′ and Rh″ which are often not available.

Several new techniques since have been developed to demonstrate these blocking antibodies by their active agglutination of Rh-positive cells in various viscous media, and these more sensitive methods have largely replaced the blocking test in routine use. Yet, there have been very few reports of further study of Rh antisera with these new techniques to determine whether blocking antibodies of other than pure Rh_0 specificity ever occur. Diamond and Abelson,[13] in describing their slide test for the blocking antibody, remarked that "Sera classified as anti Rh_0 are capable (by this technique) of agglutinating Rh′ cells." Callender, and associates[14] also have described a serum with a blocking antibody of anti-c (Hr′) specificity. In neither case, however, did the authors pursue the matter further.

It is then of interest to report the findings in an analysis of the anti-Rh sera encountered in our survey of transfused patients. Sera found to agglutinate Rh_1Rh_2 cells in albumen were tested for specificity by the same technique, using Rh_0, Rh′, and Rh″ cell suspensions in albumen. The findings on the first ten sera are summarized in Table II.

TABLE II. SERA OF TRANSFUSED MEN; TITERS WITH VARIOUS TYPES OF CELLS IN SALINE AND 30 PER CENT BOVINE ALBUMEN

SERUM	CELL TYPE					
	Rh_0		Rh′		Rh″	
	SALINE	ALBUMEN	SALINE	ALBUMEN	SALINE	ALBUMEN
1	—	1:4	—	1:1	—	—
2	—	1:64	—	—	—	—
3	—	1:2	—	—	—	—
4	—	1:8	—	1:1	—	—
5	—	1:32	—	1:1	—	—
6	—	1:64	—	1:4	—	1:1
7	—	1:8	—	—	—	—
8	—	1:4	—	—	—	—
9	—	1:5120	—	1:1280	—	—
10	—	1:32	—	1:1	—	1:1

It may be seen that, in spite of their complete lack of activity in saline, six of the ten sera had unmistakable activity against Rh′ cells in albumen, and two against Rh″. These reactions were checked with the rabbit antihuman globulin technique of Coombs and associates,[15] with excellent agreement.

Furthermore, it was possible to demonstrate that Serum 9, with the highest titer of anti-Rh′ antibodies, was capable of blocking Rh′ cells in saline so that subsequently they could not be agglutinated by a standard anti-Rh′ serum.

In order to determine whether these findings would be limited to patients sensitized by transfusions, ten unselected sera from Rh-negative women who had been sensitized by Rh-positive pregnancies were evaluated similarly. The findings are summarized in Table III.

TABLE III. SERA OF SENSITIZED RH-NEGATIVE WOMEN; REACTIONS WITH VARIOUS TYPES OF CELLS IN SALINE AND 30 PER CENT BOVINE ALBUMEN

	CELL TYPES					
	Rh_0		Rh′		Rh″	
SERUM	SALINE	ALBUMEN	SALINE	ALBUMEN	SALINE	ALBUMEN
11	—	++	—	—	—	—
12	—	++++	—	++++	—	—
13	—	++++	—	—	—	—
14	++++	++++	—	++++	—	—
15	++++	++++	+	++++	—	—
16	—	++++	—	++++	—	—
17	+	++++	—	++++	—	—
18	—	++++	—	++++	—	++++
19	++++	++++	—	++	—	—
20	++++	++++	+	++++	—	—

It may be seen that whereas the usual Rh′-typing serum consists of a mixture of Rh′ agglutinins and Rh_0-blocking antibodies, quite the opposite situation is illustrated by Serum 14, a standard Rh_0 agglutinating serum which proved to contain Rh′-blocking antibodies as well. Considering the twenty sera examined, of the eighteen which had no activity against Rh′ cells in saline, eleven (or 60 per cent) agglutinated these same cells in albumen, while two others with weak Rh′ agglutinins clumped these cells very much more strongly in albumen. While none of the twenty showed Rh″ agglutinins in saline, three (or 15 per cent) agglutinated Rh″ cells in albumen.

It is realized that this series is small, but these preliminary observations appear sufficient to cast considerable doubt on the oft repeated statement that blocking antibodies are always of Rh_0 specificity.

SUMMARY

Two popular opinions regarding the Rh factors are discussed with evidence which suggests that they are fallacious:

1. *Only a small percentage of Rh-negative persons can become sensitized by transfusion with Rh-positive blood.* Evidence is presented that more than 50 per cent of such persons may develop antibodies. It is concluded that Rh-negative individuals should be transfused only with Rh-negative blood.

2. *Blocking antibodies are always of Rh_0 specificity.* Twenty antisera from Rh-negative persons sensitized by transfusion or by pregnancy were examined for activity against Rh′ and Rh″ cells in albumen and in saline. Although none caused agglutination in saline, Rh′ antibodies active in albumen were demonstrated in 60 per cent and Rh″ antibodies in 15 per cent.

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PROGRESS

METABOLIC COMPLEXITIES OF PELLAGRA

PHILIP HANDLER, PH.D.
DURHAM, N. C.

THE current concepts of metabolism in pellagra commenced with the identification[1] of niacin as the blacktongue preventive principle of liver whose existence had been established by Goldberger and Wheeler[2] and the occurrence of this compound as part of codehydrogenases I and II.[3, 4] The numerous investigations reported since these papers have added many pieces of information, but the over-all picture is one which has grown steadily more perplexing. Although the fundamental significance of the pyridine nucleotides in cellular respiratory mechanisms is beyond dispute, attempts to correlate the behavior of such systems with the usual manifestations of niacin deficiency have met with no success.[5, 6] However, it does seem likely that failure of these systems is involved in the development of anemia in niacin-deficient dogs.[7]

The nature of the precipitating factor which results in blacktongue crises, characterized morphologically by lesions in the mucosa of the gastrointestinal tract and chemically by acidosis and dehydration, is as yet unknown. This crisis usually occurs within forty to sixty days after an adult dog is placed on a diet containing corn meal and the animal dies within a few days. Yet, the entire symptom complex disappears after a week of vigorous parenteral saline therapy, frequently never to return, although the animal survives for another three to six months.[6] While the niacin and coenzyme concentrations of the tissues of dogs in blacktongue are not reduced materially below normal, those of dogs successfully treated with salt solution, which survive several months longer, are dramatically reduced and death at this point does seem to be related to coenzyme deficiency. This situation scarcely seems compatible with simple niacin deficiency as the sole etiologic factor in the pathogenesis of blacktongue. It has been noted that the niacin content of the diets on which pellagra and blacktongue develop are not really remarkably low in niacin but contain an amount equal to that which must be added to prevent disease. Considering the rapid development of these diseases, this is a most unusual behavior for a simple vitamin deficiency. When it was found that the buccal manifestaitons of blacktongue did not appear for many months in dogs on a synthetic ration very low in niacin content, that when they did appear they were not accompanied by acidosis or dehydration, and that one-third of the dogs simply declined steadily and died after three to seven months in much the same manner as dogs on corn meal rations who have been successfully treated with salt solution, it was suggested by the present author[8]

From the Department of Biochemistry, Duke University School of Medicine.
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that corn is of positive etiologic significance in the development of blacktongue (and, presumably, of pellagra) and not merely a food of relatively low niacin content. While this was a revival of a time-honored, but hitherto unproved, thesis, it was based on experimental evidence. The same suggestion had been made earlier by Aykroyd and Swaminathan[9] after an appraisal of the niacin intake of pellagrous and nonpellagrous populations.

A new approach to this problem was offered by the finding that a suitable admixture of corn grits and a synthetic ration resulted in a suppression of rat growth which could be overcome by niacin[10] or by tryptophane.[11] At this writing, data are available concerning the effects of tryptophane on apparent niacin deficiency only in the rat and chick. This is particularly unfortunate since these findings in the rat make it difficult to understand the development of fatal niacin deficiency in the adult dog on purified rations, providing what would seem to be an otherwise adequate tryptophane intake (22 per cent casein)[8] and the use of almost identical rations as the basis for a niacin assay with pups.[12] Since tryptophane administration results in an increased urinary excretion of niacin derivatives, particularly N^1-methylnicotinamide, in rats,[13] the possibility exists that tryptophane may serve as a precursor for niacin synthesis.

The presence of considerable quantities of a substance which has not been identified positively but with properties suggestive of quinolinic acid (pyridine, 2,3-dicarboxylic acid) in rat urine after tryptophane administration[20] offers a clue to the possible pathway of such a biologic synthesis of nicotinic acid from tryptophane. The observation that pyridoxine-deficient rats do not excrete extra N^1-methylnicotinamide when given tryptophane[14] but do excrete abnormal quantities of xanthurenic acid,[15] a derivative of tryptophane, also makes a metabolic relationship between niacin and tryptophane seem likely. This defect in the metabolism of tryptophane in pyridoxine-deficient rats may be related to the demonstrated functioning of pyridoxine derivatives in decarboxylase[16] and transaminase systems.[17] This niacin-tryptophane-pyridoxine relationship seems all the more significant when one notes that there exists reason to suspect that the fluorescent compound, F_1, whose excretion increases with the development of pellagra[18] may be the pyridoxic acid found in the urine of normal individuals after the administration of pyridoxine.[19]

It is somewhat disconcerting to note that increasing the casein intake of rats does not increase the N^1-methylnicotinamide excretion in their urine.[14] However, this situation yet may provide an explanation for the situation just described, in which fatal niacin deficiency was produced in adult dogs on a diet containing 22 per cent casein and no corn meal. This recalls the failure of Goldberger and Tanner to treat successfully persons with pellagra with as much as 90 Gm. of casein per day.[21]

Since the "yield" of niacin derivatives in rat urine after tryptophane administration is quite low on a percentage basis, the possible conversion of tryptophane to niacin can be established best by a tracer experiment involving the administration of tryptophane labeled with isotopic carbon or nitrogen. The work of Krehl and associates[22] offers an alternative, albeit not mutually exclusive, mechanism for the tryptophane-niacin relationship. They observed

that niacin administration increased the efficiency of tryptophane utilization on a corn grits-low casein diet from 30 to 70 per cent. Similar studies with pyridoxine would probably yield a qualitatively similar result. Indeed, such a relationship might be demonstrable between each member of the B complex and the essential amino acids, although the present instance may operate via a specific metabolic path.

Of potentially great significance in this connection and elsewhere is the observation[23] that, like corn meal, the addition of gelatin or a tryptophane-free hydrolysate of fibrin to a low casein (9 per cent) ration markedly depressed the growth of rats and this could be prevented by the further addition of niacin or tryptophane. One explanation which suggests itself is that by improving the supply of other essential amino acids the relative deficiency in tryptophane is more marked and that such a situation is more deleterious to the animal's well-being than the original state of affairs. However, this would not account for the action of niacin. It may be that the increased over-all protein metabolism increases the demand for niacin much as an increased carbohydrate metabolism increases the demand for thiamine. There are, of course, other analogies to this situation, but it certainly suggests that an evaluation of the factors leading to the development of pellagra must consider not only the tryptophane content of the diet but the total amount of protein as well. That corn protein is tryptophane deficient has long been recognized and has been considered as a possible etiologic factor in pellagra by many investigators including Wilson, Mitchell, and Goldberger. However, no suggestion of a specific protein-tryptophane-vitamin P-P relationship was made in the literature until the recent work of the Wisconsin laboratory.[10, 11] Further investigation of the metabolic interrelationships of pyridoxine, tryptophane, niacin, and dietary protein is essential to the establishment of a rational concept of the etiology and pathogenesis of pellagra.

Complex as these interrelationships now seem, to the situation must be added the existence of toxic principles in corn and possibly other cereals. While the older literature contains many such speculations, no such material had been isolated until 1933 when Stockman and Johnston[24] reported that they had obtained from corn feed meal (corn from which the starch and oil had been removed) an acid which gave an insoluble lead salt and an easily crystallizable and soluble sodium salt. No attempt to establish the identity of this substance was made. Single doses of about 1 Gm. given to Rhesus monkeys resulted in drowsiness and mild paresis. Repeated doses produced drowsiness, general paresis, paralysis, coma, and death. On histologic examination the only significant lesions were found in the nervous system. They consisted largely of degenerative changes not unlike those reported from time to time as occurring in persons with pellagra, and more recently in dogs.[25] Guinea pigs responded in the same fashion but rabbits seemed resistant to the substance unless very large doses were employed, in which event they died after a few hours. It is unfortunate that no studies were made with dogs. A similar material was obtained from oats and from one batch of rye but not another. Considering the symptomatology reported, it is interesting to speculate upon the possible relationship of this material to the toxic principles responsible for favism, lathyrism, and "non-

convulsive ergotism'' (which seems to be related to the ingestion of large quantities of rye bread uncontaminated with ergot) and the therapeutic and prophylactic use of niacin in these states.

Woolley[26] has presented a preliminary report in which is described the isolation from corn meal of an organic base, perhaps a pyridine derivative, which inhibited the growth of mice when added in small concentration to a synthetic ration. Such a substance may behave as a niacin (or, perhaps specifically, niacinamide) antagonist in the manner already described for beta-acetopyridine.[27]

A most recent pertinent report in this regard is that of Kodicek and associates[28] who observed that the inclusion of 1.5 mg. per cent of indole-3-acetic acid (the plant hormone, heteroauxin) in a 10.5 per cent casein diet suppressed rat growth, and this could be prevented by niacin or tryptophane fed at 1 and 20 mg. per cent, respectively. Since the corn meal content of indoleacetic acid has been estimated variously as between 20 and 100 mg. per kilogram,[29, 30] it seems possible that this may be the long sought, toxic principle of corn. Further, since both the auxins and the corn toxin of Stockman and Johnston was obtained by initial heavy metal precipitation, it may be that these authors obtained a crude preparation of indoleacetic acid thirteen years ago, although they failed to identify it as such. Woolley's pellagrenic agent is an organic base which can be extracted from an alkaline medium with chloroform and so cannot be indoleacetic acid, although the possibility remains that it is a basic precursor or bound form of heteroauxin.

The excretion of indole derivatives in pellagrous urine has long been known. These have included indican[32, 33] and indolethylamine.[34] While indoleacetic acid itself has not been isolated, it is known that the red pigment observed in the urine of persons with pellagra and blacktongue dogs, thought to be a porphyrin by Beckh and co-workers,[35] is a mixture of urorosein and a substance similar to indirubin.[36, 37] While nothing is known of the indirubin-like pigment, save for its indole nucleus, urorosein is actually excreted as uroroseinogen which was shown by Herter[38] to be identical with indoleacetic acid which is converted to the red pigment urorosein on standing in strongly acid solutions in the presence of traces of nitrite or other oxidizing agents.

Until recently we had considered the presence of these indole compounds the result of unusually active bacterial putrefaction in the bowel due to the serious disturbances of gastrointestinal function occasioned by pellagra. The demonstration of a tryptophane-nicotinic acid relationship[10, 11, 13, 14, 22, 23] then seemed to indicate a revision of this concept, although the presence of excessive quantities of indole derivatives in the urine of subjects on a purportedly tryptophane-deficient diet appeared irrational. The presence of such large quantities of heteroauxin in corn meal renders such reasoning unnecessary. However, it should be realized that the urine of normal rats on a synthetic diet gives a positive urorosein reaction roughly proportional to the level of dietary casein and greatly intensified by feeding extra casein or tryptophane.[38] If this reaction is due solely to indoleacetic acid (no other known derivative of trypto-

phane gives this reaction), this substance which cannot be further metabolized[38] appears to be a normal product of mammalian tryptophane metabolism.

Much work remains to be done to establish the mode of action of indoleacetic acid in order to determine its role in the etiology of pellagra. On purely structural grounds one might conceive readily of it as an inhibitor of the metabolism of either nicotinic acid or tryptophane or both. Does it block the conversion of tryptophane to niacin, inhibit coenzyme synthesis from niacin or niacinamide, or the conversion of niacin to niacinamide or vice versa, or perhaps stimulate the metabolism of mammalian cells as it does certain plant cells and thereby increase the niacin requirement as previously postulated for the effect of administration of tryptophane-deficient proteins? This might be clarified by studies with synthetic plant hormones (such as, 2.4 dichlorophenoxyacetic acid) which contain no indole nucleus. Dubos[57] has reported that indoleacetic acid and a number of related substances inhibit the growth of *Mycobacterium tuberculosis, Streptococcus hemolyticus, Streptococcus salivarius,* and *Shigella paradysenteriae,* and this inhibition of growth can be reversed by tryptophane in a concentration about ten times that of the inhibiting auxin. If this phenomenon represents a structural inhibition similar to those of the vitamin analogues studied by Woolley, it is most unusual to find the inhibitor functioning at a lower concentration than the normal metabolite and suggests a more specific role for the auxins. It is unfortunate that no study was made of nicotinic acid in this connection. On the other hand, in similar studies in this laboratory[39] we have not observed any inhibition of growth or acid production by indoleacetic acid in cultures of such variegated organisms as *Streptococcus faecalis, Lactobacillus arabinosis, Staphylococcus aureus, Proteus vulgaris, Aerobacter aerogenes,* and *Escherichia coli,* except when rather high concentrations of indoelacetic acid were employed. Then, however, the inhibition was not alleviated by niacin or tryptophane. These facts, coupled with the failure of indoelacetic acid to stunt the growth of almost half the rats in the study of Kodicek and associates,[28] warrant considerable caution in evaluating the role of this compound in the etiology of pellagra.

The final thread in this story, to date, is the observation of Raska[31] that administration of 0.4 Gm. of adenine plus sodium phosphate per day to dogs on a good, standard laboratory ration induced the development of a blacktongue-like syndrome accompanied by a moderate hypertension in as little as one week, the animal dying after fourteen days. Until this observation is coupled with a therapeutic or prophylactic trial of niacin, tryptophane, or even whole liver substance and until some data are available concerning the quantitative distribution of adenine in foodstuffs, it will not be possible to determine the significance of this observation in the etiology of pellagra. However, this finding well may be of far-reaching fundamental significance, and further investigation should certainly be prosecuted vigorously.

Therefore, it appears that pellagra, as it occurs endemic among a corn-eating population, is a disease involving the metabolic interrelationships of niacin, pyridoxine, tryptophane, indoleacetic acid, perhaps adenine, and at least one other unidentified toxic substance, to say nothing of the effects of the numerous other vitamin and amino acid deficiencies and general level of protein intake

associated with such a diet. Which, if any, of these factors other than the niacin is involved in the development of the fatal acidosis and dehydration of the crises of blacktongue and pellagra, as seen on a corn diet and the mode of action of niacin in this connection, remains obscure. Despite these many gaps in our comprehension of the pathogenesis of pellagra, to Harriette Chick[40] must go credit for what now seems the first proper formulation of pellagra; she states that "Pellagra is caused by a toxic substance derived from the maize diet, which can be corrected by sufficient 'good' protein or perhaps by sufficient vitamin B_2 (which is found to accompany the 'good' proteins)."

That corn is not essential to the pathogenesis of classical pellagra long has been known and has been described in epidemic proportions among the inhabitants of a Japanese prison camp[41] who were living on a diet high in carbohydrate (on a percentage basis) but low in calories, protein, sources of the B complex and entirely lacking animal protein. The pellagra was complicated by nutritional edema and beriberi, in contradistinction to the dehydration of pellagra seen among corn eaters, and consisted mainly of extensive lesions of the skin and the mucosa of the gastrointestinal tract. In this connection it is well to note that there exists, as yet, no correlation between the known physiologic and chemical mechanisms involving niacin and its derivatives and the integrity of the skin and mucosa. Nor is there any explanation for the observation that exposure of the skin to sunlight not only results in an exacerbation of the dermatitis but of the systemic manifestations of pellagra as well.[42] The pathogenesis of the Chittenden-Underhill syndrome[43, 44] needs further study. This syndrome which occurs in vitamin A-deficient pups may actually bear no etiologic relation to blacktongue and may be simply another expression of the disturbed "integrity" of mucosal and epithelial structures usually found in vitamin A-deficient animals. In this case it is manifest as an infection of the mouth with Vincent's organisms as also has been found in niacin deficiency blacktongue[45] but, perhaps for entirely different reasons.

A few other unsolved problems of niacin metabolism also may be worth mentioning. No explanation is available for the fact that niacin administration results in an elevated red cell concentration of the pyridine nucleotides while nicotinamide administration does not.[46, 47] This seems even more puzzling, considering the vitamin action of nicotinamide, the existence of nicotinamide and not the acid in the coenzyme molecules, and the fact that the tissue (including erythrocytes) enzyme which destroys these nucleotides does so by cleaving nicotinamide as such from the remainder of the molecule,[48] a reaction which is inhibited by the presence of nicotinamide but not niacin.[48, 49] In contrast, it is nicotinamide which has been reported to prevent cobalt-induced polycythemia in rabbits.[50] No information is available concerning the effects of niacin in this connection nor of the effects of niacin, niacinamide, cobalt, or combinations thereof on the pyridine nucleotide concentrations of red cells in this species. While niacin and niacinamide distribute themselves equally between erythrocyte and plasma water,[47] red cells show a considerable avidity for N^1-methylnicotinamide, the erythrocyte concentration of the latter remaining considerably

elevated long after the plasma concentration has returned to normal following intravenous administration.[51]

Najjar and associates[53] have proposed that it is the methylated pyridinium form of niacin which may be the physiologically active form of the vitamin. They claim to have demonstrated antiblacktongue activity for N^1-methylnicotinamide in dogs and also to have treated successfully a hamster, moribund in nicotinic acid deficiency.[60] (It may be noted parenthetically that other workers have failed thus far to produce nicotinic acid deficiency in the hamster.) This hypothesis was furthered by the report of Vance [54] describing amelioration of dermatitis and glossitis in a case of pellagra on treatment with N^1-methylnicotinamide. However, since the psychomotor disturbances in this patient apparently became even more severe after this treatment and since the patient had previously been given 20 mg. of nicotinamide, it is not possible to evaluate properly this report. More recently, Najjar and Deal[58] have claimed to demonstrate demethylation of N^1-methylnicotinamide by estimating its lipotropic activity in choline-deficient rats. However, the variable activity they obtained undoubtedly can be ascribed more properly to the diminished appetite of such rats; this, indeed, has been found to be the case in this laboratory.[56] The minute excretion of nicotinamide and nicotinic acid in the urine of these rats after eating diets containing 1 and 2 per cent of N^1-methylnicotinamide easily may have been due to impurities in the N^1-methylnicotinamide preparation. At any rate, useful demethylation does not appear to be a major metabolic pathway for this compound. Since this substance possessed no antiblacktongue activity when tested in the Wisconsin laboratory,[55] there is, as yet, little reason to believe that the methylated form of nicotinamide is the biologically active structure, although the methylation of nicotinamide yet may be found to serve a physiologically "useful" role. Further work also is required to elucidate the physiologic significance of the oxidation of N^1-methylnicotinamide to the corresponding 6-pyridone.[52] No physiologic role has been ascribed to unbound niacin (that is, niacin not present as part of the pyridine nucleotides) which has been found in significant concentration only in the liver and whose concentration does not drop markedly even in animals dying of niacin deficiency, while that of the pyridine nucleotides is drastically reduced. This problem may be resolved by more accurate determinations of pyridine nucleotides in fresh liver.

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NITROGEN BALANCE

PHILIP HANDLER, PH.D.
DURHAM, N. C.

STUDIES of nitrogen balance have been made in countless physiologic and pathologic conditions. The scheme given herein, while obviously inadequate, is offered as a framework for the consideration and interpretation of such data.

The proteins referred to are the tissue proteins in general. While one might consider the liver proteins to be most important, it has been amply demonstrated that in hemorrhagic shock there occurs a liberation of amino acids from the proteins of skeletal muscle.[1] The processes involved in reactions 1 and 2 are but little understood. Nevertheless, the classical work of Schoenheimer and colleagues[2] has left no doubt that this interchange is of a rapid, dynamic, and constant nature heretofore totally unrealized. There must exist a host of factors which influence the rate, extent, and equilibrium point of such reactions. Reason does exist to suspect that these reactions do not necessarily entail complete degradation and resynthesis of the tissue protein molecules. A deficiency of a single essential amino acid, even in the adult expected to be in nitrogen equilibrium, would suffice to maintain the equilibrium in favor of reaction 2 since there is no reason, at present, to suspect the synthesis of a protein molecule lacking any one of its normal constituent amino acids. Interruption of endocrine control (the anterior pituitary) perhaps may shift the reaction toward amino acid accumulation as may also excessive adrenocortical or thyroid activity, while excessive pituitary function and testosterone administration in both sexes increase nitrogen retention and protein accumulation. Since the reactions involved in protein synthesis are endergonic it should be expected that any disturbance of intracellular energy metabolism, the simplest being anoxia, would also interfere with protein synthesis and result in accumulation of amino acids. All told, the mechanisms involved in reactions 1 and 2 and the physiologic factors governing them await considerable investigation.

Reaction 3 represents the reversible formation and degradation of nonprotein materials. There are only a few known examples of this type, such as glutathione, and they may be ignored in the remaining discussion. Reactions 4 and 5 represent the reversible oxidative deamination and transamination of amino acids. Again, the exceedingly rapid and constant rate of these reactions has been demonstrated by Schoenheimer and colleagues. However, the succeeding steps, urea formation (reaction 6) and ultimate CO_2 production (reaction 14), are not reversible. Any process which accelerates either of these reactions will automatically accelerate the other as reaction 5 then is no longer possible. The increased demands for energy or glucose formation imposed by such conditions as total inanition, partial caloric starvation, and diabetes may be regarded as examples of negative nitrogen balance due to acceleration of reaction 11. These

From the Department of Biochemistry, Duke University School of Medicine.
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conditions may be under nervous or endocrine control or may be simply the result of driving a reaction "to the right" by removing one of the end products. No circumstance is known in which one can definitely attribute negative nitrogen balance to increased urea synthesis, although this remains a possibility in some forms of negative nitrogen balance resulting from trauma, and in fasted animals after administration of adrenocortical extract. However, were such a situation to arise it could, perhaps, be demonstrated and alleviated temporarily by administration of purine drugs (caffeine, theobromine) which inhibit urea formation by way of the ornithine cycle but not the glutamine mechanism.[3]

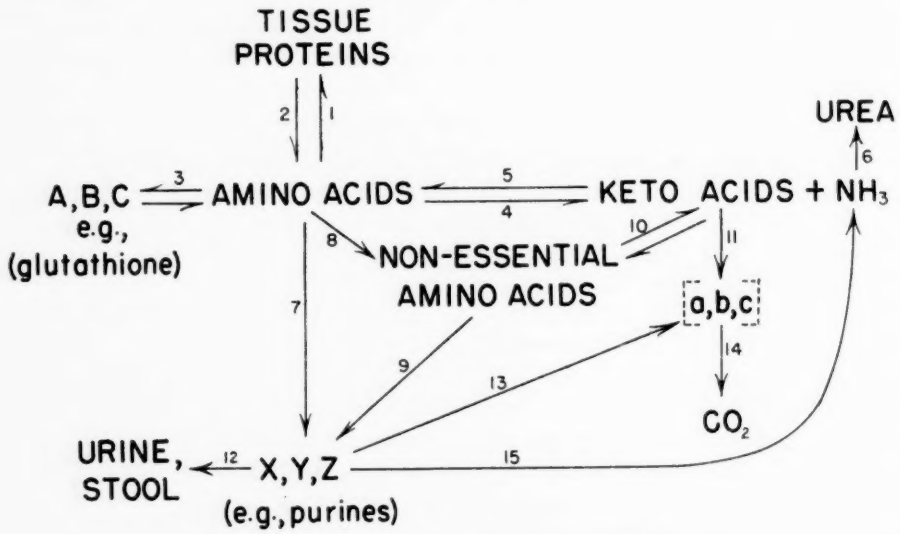

Fig. 1.—The over-all outlines of nitrogen metabolism.

Reaction 7 is the irreversible formation of nitrogenous derivatives of amino acids, that is, melanin, adrenalin, histamine, creatine, sarcosine, choline, β-alanine, carnitine, pyrrol compounds, purines, pyrimidines. It may be assumed that when these substances are formed the carbon skeletons of the essential amino acids from which they are derived are irretrievably lost for purposes of protein synthesis. In the same fashion the synthesis of nonessential amino acids from essential precursors (cystine from methionine, tyrosine from phenylalanine), reaction 8, also results in the irreversible loss of the essential amino acids involved. The apparent resynthesis of essential amino acids from nonessentials via reactions 8, 10, and 5 does not occur since, of course, entirely different α-keto acids are involved.

While little is known of the factors involved in positive nitrogen balance, there seems little reason to believe that it is the consequence of the complex phenomena necessary to dam reactions 4, 7, 8, etc. It would seem more reasonable to consider positive balance the result of a displaced equilibirium of reactions 1 and 2, perhaps with the growth hormone of the anterior pituitary as the dominant controlling factor. Again, no definite evidence is available

to decide whether this operates by decelerating protein hydrolysis (reaction 2) or accelerating its synthesis (reaction 1). However, the studies of Schoenheimer suggest that consideration is necessary only of altered reaction rates as both processes are in constant flux in young growing animals as they are in adults.

Negative nitrogen balance has been observed in many different circumstances. The situation to be considered first is that in which an adult subject is offered a diet more than adequate in all known respects but completely lacking protein or amino acids. The nitrogen excretion in adult human beings under such circumstances has been variously estimated at .025 to .04 Gm. per kilogram per day (2 to 4 Gm. per adult) of which a relatively small fraction is urea nitrogen. This value, then, represents the daily obligatory nitrogen loss and is the resultant total of all the obligatory irreversible reactions involved in the previously mentioned scheme. It is identical with what Folin termed endogenous nitrogen, although the endogenous-exogenous concept is invalid under all other circumstances.[2] It is not possible to determine to what extent the urea excretion is simply the consequence of reactions 4 and 6 or whether it is largely due to reactions 7, 8, 9, etc. The low rate of urea excretion suggests that the latter possibility is the more likely.

The second situation is the negative nitrogen balance associated with a deficiency of a single essential amino acid. It is obvious that in a serious deficiency of this sort no new protein synthesis is possible, as incomplete proteins are unlikely, and the bulk of the dietary nitrogen so provided would be discarded in the urine in various forms but largely as urea. However, there then still exists an obligatory metabolism of the missing amino acid. It is readily available from reaction 2. Some of that amino acid must then be metabolized via the paths of reactions 4, 7, 8, and 11. But, as a result, the other amino acids originally present in the protein from which this "catabolized" amino acid was derived are no longer useful for protein synthesis and are in consequence degraded and discarded. The extent of negative nitrogen balance is, therefore, far greater than that due to the loss of the nitrogen in the missing amino acid alone.

Were this description of the situation complete, negative nitrogen balance in single amino acid deficiencies should never be greater than that seen in the total protein deficiency previously discussed. In the latter situation the amount of nitrogen lost should be determined by that amino acid whose obligatory metabolism is greatest. Such data are not available yet for the ten possible deficiencies in order to determine which acid is the limiting factor, and this will be possible only when carefully controlled determinations are performed using the same subjects for all deficiencies. The same subjects also should undergo complete protein deficiency on an otherwise adequate diet, and in each case sufficient time must be allowed for the new equilibrium to be attained as the adjustment to a new "plane of protein nutrition" appears to be a rather slow process. However, reason does exist to suspect that the negative nitrogen loss may be greater in single amino acid deficiencies than in total protein depriva-

tion. While many other examples may exist in the literature, two may be taken from recent publications. Thus, it has been found that the addition of 6 per cent gelatin or a tryptophane-free fibrin hydrolysate to a diet containing 9 per cent casein actually markedly depressed the growth of young rats to a level below that on the basal 9 per cent casein diet.[4] Growth was restored to its original level by the further addition of tryptophane (or nicotinic acid), seemingly indicating that an increased total amino acid ingestion also increased specifically the tryptophane or nicotinic acid requirement. The failure of tryptophane plus gelatin to increase the growth rate above that on the basal diet is taken to indicate another amino acid as the limiting growth factor. Earlier, Jackson and associates[5] considered the rat growth inhibition occasioned by the addition of gelatin to a high casein diet, the result of a toxic property of gelatin, since growth could not be restored by addition of the amino acids known to be lacking in gelatin. This does not appear to be the case in the work with fibrin hydrolysates and further work is required before the status of gelatin can be certain. Similarly, Kornberg,[6] investigating the nutritive requirements for granulocyte production in the rat, found that the addition of methionine and tryptophane to a basal gelatin diet actually increased the mortality rate from 25 to 90 per cent in a four-day test period. Reasoning from these two examples, then, it seems not unlikely that, in like fashion, single deficiencies may induce a greater nitrogen loss than that of total protein deprivation. If this proves to be true, no mechanism for the phenomenon is yet available. The situation, however, is analogous to that long known to investigators of B complex deficiencies, namely, that single vitamin deficiencies (such as thiamine) result in a more dramatic weight loss and earlier deaths than do diets devoid of all members of the B complex. The physiologic factors involved in such deficiencies may be elucidated by more complete investigation of the mechanisms involved in the toxicity of single amino acids, virtually all of which are lethal when fed or given parenterally in large quantities.[21]

Few quantitative data are available concerning the extent of nitrogen loss in human single amino acid deficiencies. Tryptophane and lysine deficiency resulted in a maximum daily negative balance of about 4 Gm. each of nitrogen,[7] while valine and methionine deficiencies resulted in maximum daily nitrogen losses of 2.9 and 1.6 Gm., respectively, per day.[8] No quantitative data were included in the report that deficiencies of threonine, leucine, isoleucine, and phenylalanine also result in negative nitrogen balance.[9] All these were tested on healthy adult male subjects on a diet providing approximately 3,000 calories and 7 Gm. of nitrogen per day. It is noteworthy that, thus far, no deficiency of a single amino acid has occasioned a nitrogen loss greater than that of total protein deprivation, although it should be added that these all have been relatively brief experiments. It will be of interest to learn the extent of nitrogen loss in the other possible deficiencies as well as the effect of increasing the total nitrogen intake. The failure of histidine deficiency to evoke a nitrogen loss under similar conditions in the same subjects[9] seems surprising in view of the high histidine content of globin and the rapid turnover of erythrocytes.

The third commonly observed situation involving negative nitrogen balance is that occasioned by various forms of trauma, such as burns, fractures, surgery, hemorrhage, and even complete bed rest. The nitrogen lost here is excreted largely as urea. (The loss through weeping exudates may be disregarded.) No critical data yet have been obtained to determine the underlying basis for this phenomenon, nor is there necessarily a common mechanism; however, until proved otherwise it is helpful to consider it of the same origin as the so-called toxic destruction of protein associated with acute infectious states. By and large, the nitrogen loss appears to be proportional to the severity of the trauma or infection although there have been numerous exceptions reported. These negative balances have been quite refractory to parenteral or dietary protein. Instead, the administration of the equivalent of about 15 Gm. of nitrogen (as protein or an amino acid mixture) has usually resulted in an almost equivalent increment in urinary nitrogen excretion. However, protein therapy has proved most beneficial during convalescence from such injuries.

Considering, then, the various possibilities in the scheme, reaction 2 seems a definite possibility in view of the marked increase in blood amino acids found in hemorrhagic shock.[10] This perhaps might be occasioned either by moderate tissue anoxia or direct adrenocortical control. It is noteworthy that anterior pituitary growth hormone does not prevent or diminish the negative nitrogen balance observed in rats with fractured femurs.[11] The possibility of tissue anoxia does not seem significant in subjects with bone fractures or those who were simply put to complete bed rest. However, here the atrophy of the immobilized limb cannot be disregarded, and whatever may control this situation, it cannot be stated whether the total negative nitrogen balance observed after fractures is any greater than that of the injured member itself, a phenomenon which also may occur on a small scale in all the skeletal muscle of the subject during complete bed rest.

Excessive urea production as the initial basis of negative nitrogen balance has not been demonstrated. If it does occur, it could be demonstrated by purine administration. When the latter depresses urea excretion, a rise in the blood concentration and urinary excretion of amino acids should be expected when excessive urea synthesis itself is not the primary malefactor. Nor has any great demand for glucose or fat synthesis after trauma been observed while the administration of glucose in febrile states has not diminished appreciably the nitrogen loss.

Reactions 7 and 8 remain untested and likely possibilities. Should trauma occasion an increased irreversible production of some substance, X, from an essential amino acid precursor, this would be tantamount to the production of a single amino acid deficiency. The extensive negative balances of burned subjects have been rather refractory to parenteral or dietary protein therapy. If negative balance were caused by such a physiologic rather than nutritional essential amino acid deficiency, then it might be presumed that equilibrium could not be restored by providing total protein equivalent to that lost as urea but only by meeting the unusual requirement for the particular amino acid in question.

Only one amino acid, methionine, has been so tested and it was found effective in reducing the negative balance of burned rats on a low protein diet by Croft and Peters.[12] Increasing the dietary protein level also prevented negative balance, even though the increased protein supplied only one-third as much methionine as the minimum effective supplement of free amino acids. The nitrogen loss in these animals was not as dramatic as that in burned human beings and also differed in that the situation provoking nitrogen loss in burned human beings is refractory to protein therapy for a considerable period after the accident. Moreover, this effect of methionine has not been confirmed in burned rats and human beings by other investigators[13, 22] including those in this laboratory.[14] Nevertheless, the theoretical significance and clinical potentialities of such therapy certainly warrant a complete investigation of this and the other essential amino acids in like fashion.

It is noteworthy also in this connection that those investigators who have sought the minimum level of protein intake which would maintain the adult in nitrogen balance usually have thought that cannibalism would be the ideal situation in that this would most nearly provide an amino acid mixture of the desired proportions. While this may yet prove true for the growing animal, it is not true necessarily of the adult since there is no evidence to indicate that the pattern of obligatory daily amino acid destruction represented by reactions 4, 6 to 9, and 11 to 14 in anywise resembles quantitatively the relative concentrations of the amino acids of the tissue proteins. For this reason, the relative proportions of the dietary amino acids for optimum nutrition in infants and children may bear little resemblance to adult maintenance requirements.

The nature and locus of the action of endocrine control of nitrogen metabolism are now entirely obscure. Certainly, further investigation of the role of the anterior pituitary, adrenal cortex, thyroid, and gonads is warranted. The possibility of nervous control has received comparatively little attention. A clue of great potential significance is offered by patients with brain injuries who have been observed by neurosurgeons to develop perforating gastric and duodenal ulcers within twenty-four hours of the brain accident. It has been well established that prolonged ingestion of low protein diets results in the development of gastric and duodenal ulcers in laboratory animals,[15-19] and Somervell[20] has called attention to the high incidence of duodenal ulcers in the population in the south of India which subsists on an extremely low protein ration. It seems possible, therefore, that such injured patients may develop a fulminating protein deficiency which in turn leads to the ulceration.* Such ulcers are not uncommon in burned patients as well and liver damage in the latter also has been found, perhaps again indicating the possibility of an induced specific amino acid deficiency. This situation might also provide a bridge between the biochemical factors (gastric acidity, pepsin, protein deficiency), the physiologic factors (action of the vagus, pylorospasm), and the unquestionable psychogenic factors in ulcer formation.

*This possibility arose in conversations with Dr. J. S. Harris, with whom studies of this situation are now in progress.

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LABORATORY METHODS

ASSAY OF PENICILLIN, STREPTOMYCIN, TRIVALENT ORGANIC ARSENICALS, AND OTHER BACTERICIDAL AND BACTERIOSTATIC AGENTS

A Technique Based on an Original Application of Mathematical Principles With Adaptations for All Assay Purposes

Edwin E. Osgood, M.D.
Portland, Ore.

With the Technical Assistance of Barbara Gamble, B.S.

A SIMPLE, rapid, accurate, flexible technique for assay of antibacterial agents has been described elsewhere.[1] In the same article nomographs are published permitting determination of results of the assay by inspection. Also in this article are given the details of the materials and equipment needed and references to previously published assay methods.

The present article is written for those investigators who wish a full understanding of the mathematical basis for the calculation, an outline of its adaptability for various purposes, an idea of the accuracy attainable by each of these adaptations, and an understanding of the effects of the various variables investigated in the course of developing the method.

Foster and Woodruff[2] reviewed the penicillin assay methods available and formulated certain criteria for an "ideal" method. While many assay methods have been published since the date of this excellent review, none have generally displaced those in use in 1943. The investigation herein described was initiated in an attempt to meet the requirements of these authors.

THE PRINCIPLE OF THE METHOD

Our observations have shown that:

1. The growth of bacteria in culture of the type used in these experiments is directly proportional to the change in optical density of the culture.

2. The optical density can be read directly on the Klett-Summerson colorimeter (scale is = 500 × optical density).

3. An accurate growth curve can be plotted using scale readings directly from the Klett-Summerson colorimeter (1 division = 20,000,000 colonies per cubic centimeter).

4. The tangent of the angle between the straight-line growth curve of a control and of antibiotic-containing culture is directly proportional to the antibiotic concentration under the conditions specified.

From the Division of Experimental Medicine, Department of Medicine, University of Oregon Medical School.

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5. This tangent can be determined from the difference in turbidimetric readings which occurs with time in each culture during the straight-line phase of growth. Therefore, only one control and one known concentration of penicillin are needed to determine many unknowns, and a determination is possible before the difference in turbidimetric readings between the control and penicillin-containing cultures is statistically significant.

When, at a later time, the difference in turbidimetric readings between the control and penicillin-containing cultures is statistically significant, this difference is proportional to the square root of the penicillin concentration; therefore, the computation may be made by both methods if desired.

STANDARD TECHNIQUE

This permits assay by each of two methods on the same sample. All techniques are described for penicillin assay first, since only minor modifications are necessary for assay for streptomycin, trivalent organic arsenicals, or other antibiotics. All manipulations are made with sterile tuberculin or 5 c.c. Luer syringes and 26 gauge needles through 70 per cent alcohol on vaccine vial caps. The 0.85 per cent NaCl used for dilutions and the bactotryptose phosphate broth for the cultures are kept in sterile vaccine vial-capped containers. All photoelectric turbidimetric readings are made with the Klett-Summerson photoelectric colorimeter, using a No. 66 filter, on a 5 c.c. volume in the vaccine vial-capped colorimeter tubes for this instrument. All concentrations, unless otherwise specified, refer to the concentration per cubic centimeter in this 5 c.c. volume. The organism found most suitable for the test is the Oregon-J strain of *Staphylococcus aureus* obtainable from the American Type Culture Collection* by request for No. 9801 or from the National Collection of Type Cultures† by request for No. 6982. After incubation until growth has reached the proper turbidity, keep the inoculated culture in an ice bath in the refrigerator. It is usable for a week.

The following photoelectric colorimeter readings are made:

A = the broth blank on each new lot of bactotryptose broth
B′ = the culture blank on inoculated broth before placed in the ice bath; should be checked just before use each day
B′ − A = turbidity due to bacterial growth in the broth culture and should be 12 to 15; 15 corresponds to a colony count of 300,000,000 per cubic centimeter

Set up a control, a known, and as many unknown photoelectric colorimeter tubes as desired, as shown in Table I. The known should contain 0.04 unit of penicillin per cubic centimeter corresponding to the addition of 0.5 c.c. of a 0.4 unit per cubic centimeter dilution of a standard penicillin preparation. Concentrated unknowns should be diluted to an estimated concentration of 0.2 to 0.4 unit per cubic centimeter and 0.5 c.c. used. On each of these tubes in rapid sequence take the following readings:

*Georgetown University, Washington, D. C.
†The Lister Institute, London, England.

B: at 0 time just before putting tubes in the 37 degree water bath; this is the culture blank
C: 1.5 hours after incubation is started
D: 2 hours after incubation is started
E: 2.5 hours after incubation is started
F: 4 to 30 hours after incubation is started, usually the next morning

Reading D may be omitted except when unusually high concentrations are anticipated or the earliest possible result is desired. Reading B is necessary only when unknowns differ widely in turbidity or color. During the interval between Readings B and C, the cultures are in the lag phase of growth, the growth curves are not straight line, and there is little antibiotic action.

D − C = y_1, the turbidity due to bacterial growth during the first half hour of straight-line growth
E − D = y_2, the turbidity due to bacterial growth during the second half hour of straight-line growth

Ideally, y_1 and y_2 should be equal.

E − C = y_3, the turbidity due to bacterial growth in the first hour of straight-line growth, = $y_1 + y_2$

The figure usually used in the calculation by Method 1 is y_3.

F − B = turbidity due to bacterial growth called corrected F, and the difference between corrected F for the control and for each of the antibiotic-containing cultures is called y_4

This is the figure used in calculation by Method 2. Data are recorded as shown in Table I.

TABLE I. DATA OF TYPICAL EXPERIMENT

		TUBE 1—CONTROL (C.C.)		TUBE 2—KNOWN (C.C.)		TUBE 3—UNKNOWN (C.C.)	
Add							
0.85% NaCl		0.5		–		–	
0.4 U. per c.c. Na penicillin		–		0.5		–	
Unknown solution		–		–		0.5	
Broth culture		4.5		4.5		4.5	
READING	TIME* (HR.)	COLORIMETER READING	DIFFERENCE Y	COLORIMETER READING	DIFFERENCE Y	COLORIMETER READING	DIFFERENCE Y
A=10, broth blank	0						
B, blank for F	0	20		21		20	
C	1.5	60		54		56	
$y_1 = D - C$			21		13		16
D	2.0	81		67		72	
$y_2 = E - D$			22		12		16
E	2.5	103		79		88	
$y_3 = E - C = y_1 + y_2$			43		25		32
F	22.5	230		51		110	
Corrected F=(F−B)		210		30		90	
$y_4 = F_1 - F_{2 \text{ or } 3}$					180		120

*In the 37° C. water bath.

THEORY OF THE CALCULATION

Note that area I in Fig. 1 shows the growth curves of a control and a penicillin-containing known or unknown of a typical experiment. Area II is an enlargement of the indicated portion of area I with a relative increase in scale

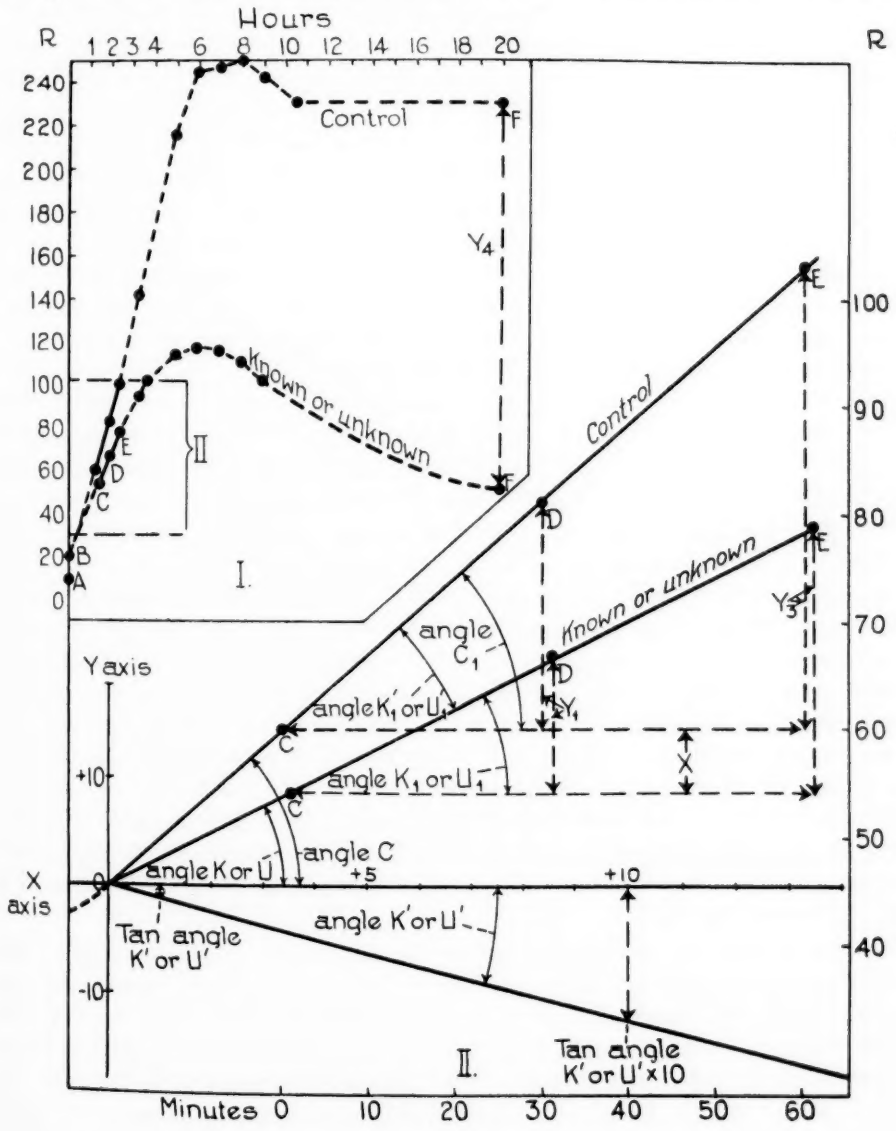

Fig. 1.—Explanation of the theory of the computation.

on the X axis to separate the curves for greater clarity. R refers to photoelectric colorimeter readings. The time scale for area I is in hours; for II it is in minutes, with the 0 beginning at the time of Reading C. In a typical experiment this is 1.5 hours after the tubes were placed in the 37° C. water bath, but

it may be any time while growth is straight line. A, B, C, D, E, and F are, respectively, the photoelectric colorimeter readings for the broth blank, the culture blank, the three readings during the 1-hour period used in the calculation for Method 1, and the final reading at 18 to 24 hours used in the calculation by Method 2. Note in the enlarged area II that the straight-line growth curves of the control and penicillin-containing known or unknown separate at the point marked 0, where the Y and X axis are drawn to intersect, and that prior to this, as indicated by the curved dotted line, the growth is not straight line and there is no difference in turbidity between the control and the penicillin-containing cultures. Note that y_1 is the difference between the colorimeter reading, D, of the control or penicillin-containing culture and its reading, C, 30 minutes earlier, and that this forms one side of a right triangle with the time interval, X, of 30 minutes, or 5 units on the X axis forming the other side and the growth curve the hypotenuse. The same is true for the difference, y_3, between the Readings C and E, except that here the value of X is 1 hour or 10 units on the X axis. Therefore, the value of y_1 divided by the corresponding value of X is the tangent of the angles C_1 or of K_1 or U_1. Since parallel lines intersecting a straight line form equal angles, angle C_1 = angle C and angle K_1 or U_1 = angle K or U. By inspection it is evident that angle C is the angle which the control makes with the X axis and angle K or U is the angle which the penicillin-containing culture makes with the X axis. It is apparent that y_1 or y_3 divided by the corresponding values of X give the tangents of C_1, K_1, or U_1 and hence of C, K, or U. Note further that while the Readings C, D, and E are taken later for the penicillin-containing known or unknown than for the control, this time lag does not alter the value of the tangents so long as the growth curves are still in the straight-line phase. Note by inspection that angle C – angle K or U = angle K'_1 or U'_1, the angle at which the penicillin-containing culture leaves the control culture. By construction, angle K′ or U′ = angle K'_1 or U'_1. This construction is equivalent to making the growth curve of the control coincide with the X axis and rotating the growth curve of the penicillin-containing culture through an equal arc. The tangent of angle K′ or U′ then is a measure of the rate at which the growth curves of the antibiotic-containing cultures are deviating from the growth curve of the control culture.

This method of computation allows for differences in effectiveness of the antibiotic agents with differences in growth rate. It has been found, as shown by the results reported in Tables IV to VI for Method 1, that the tangent of the angle between the growth curves of the control and the known or unknown cultures is directly proportional to the antibiotic concentration for the antibacterial agents so far tested. Because it is so inconspicuous in the diagram at the point where $x = +1$, the numerical value of 10 x tan K′ or U′ also is shown in Fig. 1 at the point where $x = +10$.

The calculation by Method 2 is based on the observation that the square root of the penicillin concentration is proportional to the difference between the photoelectric colorimeter readings due to turbidity from bacterial growth of the control culture and the penicillin-containing culture after 4 to 6 hours of incubation. Since this difference increases with time, computations by this method

increase in accuracy as long as the difference increases. Computation by Method 2 may be useful at 3 to 6 hours if inadvertently a higher concentration of penicillin was introduced than intended and the y_1 readings were already beyond the straight-line phase.

CALCULATION

An example of the calculation is given in Table II.

Method 1.—Let x = time in 0.1 of an hour or 6-minute intervals and y = the difference between Readings D or E and Reading C in the same tube.

$$\tan C = \frac{y}{x} = \frac{y_1}{5} \text{ or, } \frac{y_2}{10}$$

TABLE II. CALCULATION FROM DATA IN TABLE I*

	TUBE 1 CONTROL	TUBE 2 KNOWN	TUBE 3 UNKNOWN
	Method 1		
1.5 to 2 hours			
y_1	21	13	16
Tangent = $\frac{y_1}{5}$	4.2 = tan C	2.6 = tan K	3.2 = tan U
Angle from tan table	76.6° = C	69.0° = K	72.7° = U
C − K or U		7.6° = K′	3.9° = U′
Tangent from tan table		0.1334 = tan K′	0.0682 = tan U′
$\frac{\tan K'}{40} = m$		$\frac{0.1334}{40} = 0.003335$	
$\frac{\tan U'}{m}$ = units × 1,000			$\frac{0.0682}{0.003335} = 20.5$
1.5 to 2.5 hours			
y_2	43	25	32
Tangent = $\frac{y_2}{10}$	4.3	2.5	3.2
Angle from tan table	76.9°	68.2°	72.6°
Angle C − K or U		8.7°	4.3°
Tangent from tan table		0.1530	0.0752
$\frac{\tan K'}{40} = m$		$\frac{0.1530}{40} = 0.003825$	
$\frac{\tan U'}{m}$ = units × 1,000			$\frac{0.0752}{0.003825} = 19.6$
	Method 2		
$f = \frac{y_4}{6.325\dagger}$	180	$\frac{180}{6.325\dagger} = 28.46$	120
$\sqrt{z} = \frac{y}{f}$			$\frac{120}{28.46} = 4.216 = \sqrt{z}$
$z = \left(\frac{y}{f}\right)^2$ = units × 1,000			$4.216^2 = 17.8$
	Summary		
Theoretical units			0.0200
Method 1 with y_1			0.0205 = + 2.5%
Method 1 with y_2			0.0196 = − 2.0%
Method 2			0.0178 = −11.0%

*In practice, since the position of the decimal point in the final result is evident by inspection, the decimal points may be ignored; however, it is necessary to carry the operations to four significant figures if the full accuracy of the method is to be attained.

†It is usually more convenient to express results in hundredths of a unit and divide by $2 = \sqrt{4}$, that is $\frac{180}{2} = 90, \frac{120}{90} = 1.333, 1.333^2 = 1.78$ hundredths of a unit = 0.0178 units per cubic centimeter.

when C is the angle which the control curve makes with the X axis, y_1 is Reading D – Reading C for the control, and y_3 is Reading E – Reading C. Determine the tangents of growth curves of each known (K) and unknown (U) from the appropriate readings on these tubes in precisely the same manner.

Look in a tangent table* and find the values for the angles C, K, and U corresponding to tans C, K, or U.

Angle C – angle K = angle K′ and angle C – angle U = angle U′, the angles between curves of the control and each penicillin-containing culture.

From a tangent table obtain tan K′ which is directly proportional to the amount of penicillin. Therefore, tan K′ divided by the units of penicillin in the known expressed in thousandths is a constant, m.

In the example given, 0.04 unit penicillin was used in the known. The factor for calculation of the unknowns is found from:

$$\text{Concentration in thousandths of units} = m \tan K',$$

hence,

$$m = \frac{\tan K'}{40}$$

The concentration of penicillin in the unknowns can now be determined by substitution of tan U′ for each unknown for tan K′ in the previously mentioned equation.

Multiply by the dilution of the unknown for the value in the original material.

ALTERNATE METHOD OF CALCULATION WITHOUT USE OF TANGENT TABLES

From the trigonometric formula for the tangent of the difference of two angles it follows that:

$$\frac{\tan C - \tan K}{1 + \tan C \cdot \tan K} = \tan K'$$

and

$$\frac{\tan C - \tan U}{1 + \tan C \cdot \tan U} = \tan U'$$

The following is an example using data for $\frac{y_3}{10}$ from Tables I and II for tans C, K, and U:

$$\frac{4.3 - 2.5}{1 + 4.3 \cdot 2.5} = \frac{1.8}{1 + 10.75} = \frac{1.8}{11.75} = 0.1532 = \tan K'$$

$$\frac{4.3 - 3.2}{1 + 4.3 \cdot 3.2} = \frac{1.1}{1 + 13.76} = \frac{1.1}{14.76} = 0.0745 = \tan U'$$

$$\frac{0.1532}{40} = 0.00383$$

$$\frac{0.0745}{0.00383} = 19.5 \text{ thousandths of a unit per cubic centimeter.}$$

*Lange, N. A.: Handbook of Chemistry, ed. 6, Sandusky, Ohio, 1946, Handbook Publishers, p. 198 (appendix, mathematical tables, and formulas); or see other handbooks or trigonometry texts.

Method 2.—Subtract the corrected Reading F for the known penicillin solutions and for the unknowns from the corrected Reading F of the control, Tube 1. Call this difference y_4. Let z equal the concentration of penicillin in the colorimeter tube in hundredths of a unit per cubic centimeter. The difference in reading is directly proportional to the square root of the concentration. Therefore, Equation 1 expresses the relationship between y and z, when f is a factor, usually between 60 and 120, corresponding to the y_4 value for 0.01 unit of penicillin per cubic centimeter and depending on the initial inoculum, the susceptibility of the organism, and the rate of growth.

$$\text{Equation 1:} \quad y_4 = f \sqrt[2]{z}$$

Determine the factor f by substituting the known concentration of penicillin (z) expressed as hundredths of units and the corrected colorimeter difference reading, y_4, in the Equation 1 and solving for f. After f has been determined, substitute the y_4 readings for each unknown and solve for the concentration z. A sample calculation is given in Table II.

Modifications for Special Purposes.—The technique previously described is suitable for the determination of penicillin in solution, in cerebrospinal fluid, in urine, and in *Penicillium notatum* cultures. Slight modifications are necessary for determination in blood serum, ascitic fluid, or pleural fluids and for greatest accuracy in urine and *P. notatum* cultures. The computation is the same for all, except that the dilution factor varies.

Directions for Blood Serum: Record the time of the last administration of penicillin. Separate the serum from a 5 to 10 c.c. blood sample collected in a rubber vial-capped centrifuge tube twenty to ninety minutes after the last dose of penicillin. Record the time of collection.

Into Tube 1, place 0.5 c.c. of sterile saline and 0.5 c.c. of the serum of the patient, taken just before dose of penicillin, or clear serum from a healthy person who has not been receiving neoarsphenamine or other trivalent organic arsenicals; into Tube 2, place 0.5 c.c. of standard penicillin solution and 0.5 c.c. of the same serum used in Tube 1; into Tubes 3, 4, 5, etc., place 0.5 c.c. of saline solution and 0.5 c.c. of each unknown serum. Add to each 4.0 c.c. of the broth culture.

If values below 0.1 unit per cubic centimeter are anticipated, it is better to use 1.0 to 2.0 c.c. of the serum and 3.5 or 2.5 c.c. of inoculated broth and to multiply by a dilution factor of 5.0 or 2.5 instead of 10 to get the units in the unknown serum.

Directions for Cerebrospinal Fluid: These are the same as for solutions of penicillin, except that larger quantities may be necessary if the concentration is low, and dilutions may be necessary if the concentration is high.

Directions for Pleural or Ascitic Fluid: Centrifugate and, if grossly purulent, pass the supernatant fluid through a Seitz filter to clarify and sterilize. Then proceed as for blood serum.

Directions for P. notatum Cultures: Make an accurate dilution of the centrifugated or Seitz-filtered* medium, so that the estimated content in the final broth culture will be approximately 0.04 unit per cubic centimeter and will certainly lie between 0.01 and 0.1 unit per cubic centimeter. Use uninoculated equally diluted *P. notatum* medium for the control and known penicillin tubes and proceed as for penicillin solutions.

MODIFICATIONS OF TECHNIQUE

Certain simple adaptations of the standard technique previously described have been found useful and are herein listed. All procedures follow this standard technique except as noted. Results shown in Tables III to VI are consecutive and unselected and include normal routine manipulative errors. Dilutions were made with unselected tuberculin syringes.

TABLE III. RESULTS WITH TECHNIQUE FOR MAXIMUM SIMPLICITY*

UNITS PER C.C.		PER CENT
ADDED	FOUND	ERROR
	0.073	– 9
	0.064	–20
	0.081	+ 1
0.080	0.047	–41
	0.049	–39
	0.052	–35
	0.096	+20
Av.	0.066	–17.5
0.060	0.047	–21
	0.051	–15
0.050	0.046	– 8
	0.040	0
	0.041	+ 2.5
	0.037	– 7.5
	0.038	– 5.0
	0.036	–10.0
	0.039	– 2.5
0.040	0.042	+ 5.0
	0.040	0.0
	0.050	+25.0
	0.040	0.0
	0.042	+ 5.0
	0.040	0.0
	0.040	0.0
	0.042	+ 5.0
	0.042	+ 5.0
Av.	0.0406	+ 1.5
0.035	0.034	– 3.0
0.030	0.027	–10.0
	0.027	–10.0
0.025	0.022	–12.0

UNITS PER C.C.		PER CENT
ADDED	FOUND	ERROR
	0.016	–25.0
	0.019	– 5.0
	0.012	–40.0
	0.014	–30.0
	0.014	–30.0
	0.010	–50.0
	0.019	– 5.0
	0.025	+25.0
	0.019	– 5.0
0.020	0.018	–10.0
	0.018	–10.0
	0.021	+ 5.0
	0.027	+35.0
	0.017	–15.0
	0.029	+45.0
	0.026	+30.0
	0.023	+15.0
	0.023	+15.0
	0.015	–25.0
Av.	0.0192	– 4.0
	0.005	–50.0
	0.005	–50.0
	0.007	–30.0
	0.005	–50.0
	0.008	–20.0
0.010	0.009	–10.0
	0.005	–20.0
	0.007	–30.0
	0.008	–20.0
	0.010	0.0
	0.006	–40.0
Av.	0.0068	–32.0

*All were computed from a single control and a single known containing 0.040 unit of penicillin per cubic centimeter. Accuracy is considerably increased if two knowns are included and the formula given under Technique for Maximum Range is used.

*Centrifugation or filtration through a Seitz filter is necessary only when research accuracy is desired.

TABLE IV. RESULTS WITH TECHNIQUE FOR EARLIEST POSSIBLE RESULT*

UNITS PER C.C. ADDED	UNITS PER C.C. FOUND	PER CENT ERROR	UNITS PER C.C. ADDED	UNITS PER C.C. FOUND	PER CENT ERROR
0.500	0.220	−66.0	0.030	0.33	+10.0
0.100	0.094	− 6.0		0.022	+10.0
	0.054	−46.0		0.030	+50.0
0.080	0.078	− 2.7		0.022	+10.0
	0.075	− 3.1		0.021	+ 5.0
	0.094	+17.5		0.026	+30.0
	0.054	−32.7		0.021	+ 5.0
0.060	0.061	+ 1.7	0.020	0.023	+15.0
	0.040	−33.3		0.030	+50.0
0.040	0.040	0.0		0.019	− 5.0
	0.040	0.0		0.023	+15.0
	0.040	0.0		0.021	+ 5.0
	0.040	0.0		0.018	−20.0
	0.031	−22.5	Av.	0.023	+15.0
	0.051	+22.5	0.040†	0.036	−10.0
	0.040	0.0		0.036	−10.0
	0.040	0.0		0.027	−32.5
	0.023	−42.5		0.036	−10.0
	0.040	0.0		0.039	− 2.5
	0.031	−22.5		0.031	−22.5
	0.040	0.0		0.043	+ 7.5
	0.040	0.0		0.039	− 2.5
	0.040	0.0		0.035	−12.5
Av.	0.0394	− 1.5	Av.	0.0358	−12.5

*All results were available within two hours.
†These were computed from 0.020 unit per cubic centimeter known, all others from a 0.040.

TABLE V. RESULTS WITH ROUTINE TECHNIQUE

UNITS PER C.C. ADDED	UNITS PER C.C. FOUND	PER CENT ERROR	UNITS PER C.C. ADDED	UNITS PER C.C. FOUND	PER CENT ERROR
0.080	0.076	− 5.0	0.0200	0.0198	− 1.0
	0.101	+25.1		0.0186	− 7.0
	0.093	+13.2		0.0189	− 5.5
	0.138	+72.5		0.0189	− 5.5
	0.054	−32.5		0.0216	+ 8.0
Av.	0.0924	+15.5		0.0275	+37.5
0.060	0.066	+10.0		0.0192	− 4.0
	0.047	−21.7		0.0206	+ 3.0
0.050	0.040	−20.0		0.0207	+ 3.5
0.040	0.045	+12.5		0.0210	+ 5.0
	0.037	− 7.5		0.0241	+ 2.5
	0.040	0.0		0.0174	−13.0
	0.044	+10.0		0.0243	+21.5
	0.032	−20.0		0.0186	− 7.0
	0.036	−10.0		0.0195	− 2.5
	0.040	0.0		0.0232	+16.0
	0.040	0.0		0.0185	− 7.5
	0.036	−10.0		0.0186	− 7.0
	0.036	−10.0		0.0196	− 2.0
	0.040	0.0	Av.	0.02056	+ 2.8
	0.036	−10.0	0.0100	0.0110	+10.0
	0.040	0.0		0.0130	+30.0
	0.040	0.0		0.0076	−24.0
Av.	0.0387	− 3.25		0.0112	+12.0
0.035	0.034	− 3.0		0.0103	+ 3.0
0.030	0.028	−6.7		0.0090	−10.0
	0.026	−13.3		0.0079	−21.0
	0.024	−20.0		0.0080	−20.0
0.025	0.024	−4.0		0.0100	0.0
	0.022	−12.0		0.0070	−30.0
			Av.	0.0095	− 5.0

For Maximum Simplicity.—Carry out all incubations in an incubator. Omit all readings except Reading F at 4 to 30 hours, preferably 18 to 24 hours, after start of incubation. Compute by Method 2 only, using nomograms.[1] Accuracy for a single reading is about ±25 per cent, as shown by examples in Table III.

For Earliest Possible Result.—After 1-hour incubation read, at 15-minute intervals, Readings C, D, and E. Calculate by Method 1 on basis of first half-hour period in which the successive 15-minute readings on the same tube agree within two points and differ from control by more than two points. Results are given in Table IV. Note that accuracy is much less at low concentrations, but the readings may be continued to follow the standard technique.

For Routine Technique.—Take Readings C and E at the beginning and end of any 1-hour period, beginning between 1.5 and 3.5 hours after the tubes are placed in the water bath. Results are shown in Table V. Accuracy of a single determination is about ±10 per cent.

To simplify laboratory procedure, inoculated broth may be introduced into the culture tubes by a pipetting machine and micropipettes or drops from calibrated needles utilized for introduction of concentrated samples.

TABLE VI. RESULTS AT EXTREMES BY TECHNIQUE FOR MAXIMUM RANGE*

METHOD 1		METHOD 2		
UNITS PER C.C.		UNITS PER C.C.		PER CENT
ADDED	FOUND	FOUND	AVERAGE	ERROR
0.256	0.245	0.145	0.195	− 20.0
0.128	0.125	0.141	0.133	+ 2.0
0.100	0.080	0.078	0.079	− 21.0
	0.087	0.084	0.086	− 14.0
0.080	0.077	0.082	0.079	− 1.2
	0.081	0.079	0.080	0.0
	0.092	0.092	0.092	+ 15.0
0.064	0.064	0.077	0.070	+ 9.4
0.060	0.062	0.061	0.061	+ 1.7
	0.057	0.064	0.061	+ 1.7
0.008	0.0081	0.0082	0.0082	+ 2.5
	0.0094	0.0148	0.0121	+ 51.0
	0.0063	0.0097	0.0080	0.0
0.004	0.0061	0.0050	0.0055	+ 27.5
	0.0020	0.0013	0.0017	− 57.5
	0.0037	0.0052	0.0045	+ 12.5
0.002	0.0019	0.0014	0.0017	− 15.0
	0.0023	0.0035	0.0029	+ 45.0
	0.0011	0.0011	0.0011	− 45.0
0.001	0.0011	0.0017	0.0015	+ 50.0
	0.0030	0.0017	0.0023	+115.0
0.0005	0.0006	0.0004	0.0005	0.0

*Accuracy in middle range is intermediate between routine technique and technique for maximum accuracy.

For Maximum Sensitivity to Low Concentrations.—Use Oxford *Staph. aureus* strain H. Insure that inoculum gives B′ – A reading of 10 to 12 or less and that the known contains a final concentration of 0.008 unit per cubic centimeter of standard penicillin. Results are shown in Table VI.

For Maximum Accuracy.—Determine the approximate concentration. Dilute, with precision-calibrated glassware, all unknowns to a calculated final concentration of 0.04 unit per cubic centimeter. Run control, known, and each unknown in quadruplicate, using the standard or routine technique and average results.

Our results by this method agree within ±5 per cent. Duplicate determinations on standard penicillin agreed within ±2.5 per cent with one control, one known, and three replicate unknowns. Inspection of the 0.04 unit per cubic centimeter block of Table V will indicate the results available by any number of replicate determinations in this concentration range.

For Maximum Range.—Use a series of knowns ranging in final concentration by 0.02 unit steps from 0.2 to 0.02 unit per cubic centimeter and by 0.002 unit steps from 0.01 to 0.002 unit per cubic centimeter. Use as knowns the nearest higher and lower concentrations, compute by both Methods 1 and 2, and average the results.

Our experience has shown that the concentration by Method 2 is more accurately given by the relation

$$y_4 = f\sqrt{z} - b$$

where b is an arbitrary constant. To utilize this formula, two knowns are needed. The following example illustrates the method of determination of constants:

For 0.040 unit per cubic centimeter	$y_4 = 192$
For 0.010 unit per cubic centimeter	$y_4 = 84$

Expressing concentration z in hundredths of units and substituting these values,

$$(1)\quad 192 = f\sqrt{4} - b$$

$$(2)\quad 84 = f\sqrt{1} - b$$

Substracting (2) from (1) $108 = f$.
Substituting $f = 108$ in equation (1) or (2).

$$b = 24$$

Hence, in this example, $y_4 = 108\sqrt{z} - 24$. Results routinely obtained by this method of computation are shown in Table VI.

TECHNIQUE FOR STREPTOMYCIN ASSAY

The method is equally satisfactory for streptomycin assay. The only modifications necessary are the use of two knowns containing streptomycin and making Reading F for Method 2 at 4 to 6 hours instead of the next day.

Knowns are prepared with a final concentration of 1 unit (1μg) and 3 units (3 μg) per cubic centimeter. Calculation by Method 1 is identical with that described for penicillin.

If Method 2 is used, the concentration is *directly* proportional to the y_4 difference in colorimeter reading; hence,

$$y_4 = f\ z$$

when z is the streptomycin concentration in units per cubic centimeter. If it be desired to read the streptomycin cultures the next day, a known of 10 units per cubic centimeter must be used. Nomograms[1] may be used for calculation.

The effective range for streptomycin is 0.2 to 20 units per cubic centimeter in the final culture. Sensitivity may be increased for low concentrations by the use of up to 50 per cent serum and correspondingly less broth culture medium.

TECHNIQUE FOR TRIVALENT ORGANIC ARSENICALS

The trivalent organic arsenicals used in the treatment of syphilis have been shown[3] to be very effective antibacterial agents against all strains of staphylococci investigated, as well as against hemolytic streptococci, some strains of *Hemophilus influenzae,* and many strains of *Streptococcus viridans.* Therefore, the method herein described may be used for the assay of any of these trivalent organic arsenicals. The technique for Method 1 is identical with that for penicillin or streptomycin and for Method 2 it is the same as that described for streptomycin, since the y_4 differences are directly proportional to the concentrations. The ideal concentration of the known for both methods with neoarsphenamine is 1.5 μg per cubic centimeter in the final culture. The ideal concentration for mapharsen is 0.75 μg per cubic centimeter. For the other trivalent organic arsenicals, the activity against bacteria is proportional to the arsenic content relative to that of neoarsphenamine,[3] so that knowns for these should be so diluted that the final culture contains 0.3 μg per cubic centimeter of arsenic. The effective range for neoarsphenamine is from 0.2 to 3.0 μg per cubic centimeter; therefore, unknowns should be diluted to an estimated concentration of 2 to 30 μg per cubic centimeter when 0.5 cubic centimeter is used with 4.5 cubic centimeter of broth culture.

DETERMINATION OF SERUM LEVELS OF ANTIBACTERIAL AGENTS DURING THE ENTIRE TIME PERIOD BETWEEN DOSES

Except when antibacterial agents are given by continuous drip, a single serum level does not indicate the concentration at any time except the moment when the blood was taken. However, the levels at other times may be roughly computated by the factors given elsewhere.[1] They can be accurately determined by making two determinations an hour or two apart, the first one 30 minutes to an hour after administration of the drug. Using the equal interval scale for time and the logarithmic scale for concentration, plot on polycyclic semilog graph paper these two points and draw a line through them. For the trivalent organic arsenicals, follow the same procedure, plotting the results on log log paper. The serum levels at any time after the initial mixing may now be read directly from the graph.

TECHNIQUE FOR QUANTITATIVE BACTERIOLOGIC STUDIES

It seems possible that this technique could be used equally well for quantitative studies of the effects of any variable on bacterial growth, and the results would be available quickly instead of having to wait for colony counts and with far greater accuracy than colony counting technique affords. Growth could be stopped at any desired stage and quantitative studies made on the medium.

THE EFFECTS OF VARYING THE INOCULUM

Any clear, fluid, bacteriologic medium may be used if light transmission is not too low. Hartley broth gives even more satisfactory results, and because it does not contain dextrose might be preferable in testing *P. notatum* cultures which may contain notatin. The bactotryptose medium was selected because it was readily available and easily prepared, is more or less standard in composition, is already buffered, and gives very rapid growth to high turbidity. Double- or triple-strength bactotryptose broth gave no better results than the standard-strength broth. Addition of serum does give better results than the standard-strength broth. One per cent and 2 per cent bactopeptone broth gave slower growth to lower turbidities. The faster the growth, the more uniform its rate, and the higher the final turbidity, the better are the results with penicillin; and the slower the growth, the greater is the sensitivity to streptomycin. For penicillin y_3 values of 30 to 70 are satisfactory, and for streptomycin y_3 values of 20 to 50 are satisfactory. Still better media no doubt can be discovered.

THE EFFECT OF VARYING THE INOCULUM

These effects are shown graphically in Fig. 2 for penicillin. The lower the inoculum, the slower is the start of the straight-line phase of growth, the more rapid is the final growth rate attained, the more sensitive is the technique for low concentrations, the more accurately do the penicillin effects correspond to the equations, and the greater are the y_4 and tangent differences per unit concentration of penicillin. However, with low inocula, a slight difference in the size of the inoculum or the blank reading of the colorimeter tube introduces a much greater error in the result, and the range for high concentrations of penicillin is reduced by early cessation of growth. Inocula corresponding to a B′ – A reading of 30 or over are unsatisfactory because of small differences in growth rate with large differences in penicillin concentration. For most purposes an inoculum corresponding to a B′ – A reading of 12 to 15 is most satisfactory.

THE EFFECTS OF VARYING THE STRAIN OR SPECIES OF ORGANISM USED

Much more work needs to be done on this. The ideal organism for routine use should be only moderately sensitive to penicillin action, should grow rapidly to high turbidities, should show no tendency to produce variants, and should remain in the straight-line phase of growth for a long time on simple and inexpensive media. The Oregon-J strain of hemolytic *Staph. aureus* more nearly fulfills these requirements than any other organism so far tested and is very satisfactory also for streptomycin and trivalent organic arsenicals. The strain of

beta hemolytic streptococcus investigated did not grow to high enough turbidities or rapidly enough; the Oxford *Staph. aureus* strain H, while slightly superior for very low concentrations of penicillin, did not stay in the straight-line phase of growth long enough and ceased growth at a turbidity reading of 200 versus 250 for the Oregon-J strain. The National Institute of Health's strain 209 P had the same defects as the Oxford strain H; however, it was not as sensitive to low concentrations, although it was more sensitive than the Oregon-J strain. Other strains of staphylococci, strains of *Bacillus subtilis*, and pneumococci have been investigated and were found unsuitable.

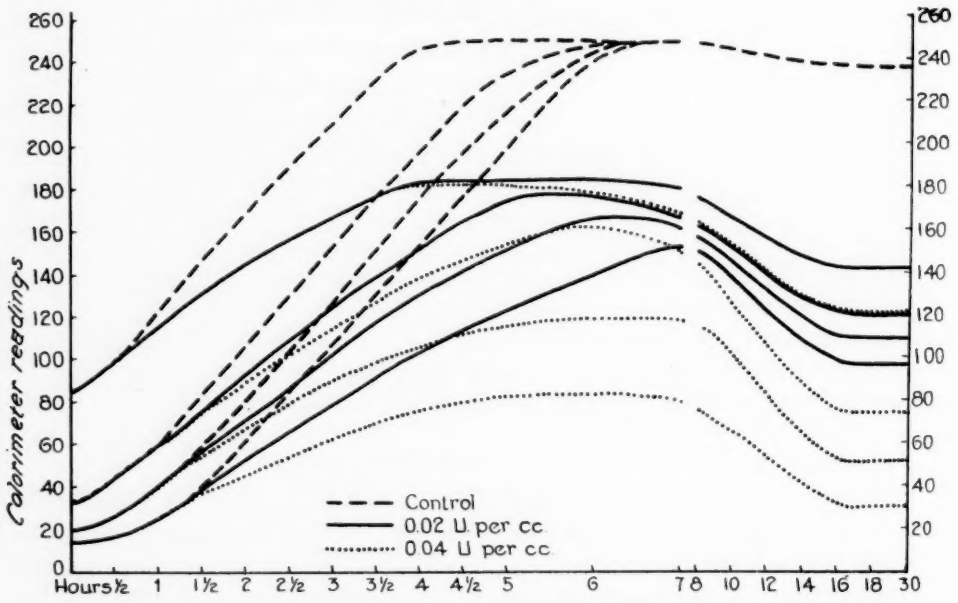

Fig. 2.—Effects of variations in size of inoculation on growth curves of the control and penicillin-containing cultures.

THE EFFECTS OF VARYING THE TIME OF READINGS

As may be seen from Fig. 2, the longer the interval between readings used in the computation by Method 1, the smaller the relative error in the photoelectric colorimeter readings, but the greater the error due to departure of the growth curve from a true straight-line curve, especially with high concentrations. The 1-hour period is best for the entire range, but a half-hour period is best for concentrations above 0.06 unit per cubic centimeter with the Oregon-J strain. Too early initial readings introduce error by including a part of the lag phase of penicillin action or of the prestraight-line growth curve, and too late readings introduce error by including nonstraight-line portions of the growth curves. With the standard technique and the Oregon-J strain, growth is essentially straight line from 1¼ to 4 hours with concentrations below 0.06 unit per cubic centimeter and departs least from a straight-line curve between 1.5 and 3.5 hours.

In calculation by Method 2, y_4 differences increase in significance and reliability rapidly from 3 hours, when they first become significant for high concentrations, to 6 hours and then slowly until 17 hours, after which they change very little in the next 24 hours.

THE EFFECTS OF VARYING THE TIME SCALE

The reason for choosing 6-minute units is that it brings the tangents into a convenient part of the tangent table and involves dividing by 10, which is merely pointing off a decimal place. It is not important that these be exact 6-minute units; equally accurate results are obtained if the time interval is somewhat shorter or longer than an hour and 10 is still used as the divisor, provided that the time interval between readings is the same for each tube.

THE EFFECTS OF VARYING THE FILTER

Any filter may be used, but the advantages of the No. 66 filter are that it transmits red and yellow light very well, giving a low blank that is little affected by the yellow colors of the medium, penicillin solutions, blood serum, urine, or the red colors of slightly hemolyzed serum. For certain research purposes, where very low concentrations are to be determined, a green No. 54 filter is preferable, since it gives a higher difference for small changes in turbidity; however, the blanks will be higher with this filter. Still greater accuracy was obtained with the blue No. 42 filter and Hartley broth, but the bactotryptose broth gives too high a blank A reading.

USE OF OTHER PHOTOELECTRIC COLORIMETERS

The principle of the method is applicable directly to any photoelectric colorimeter having a logarithmically spaced scale and can be adapted to other types by the formula* $(2.000 - \log_{10}T) \times 500 \times \frac{12}{\text{inside tube diameter in mm.}} =$ reading on Klett-Summerson instrument. However, the Klett-Summerson instrument is strongly recommended because of the convenient size of the tubes, the large number of scale divisions, the great rapidity with which readings may be made, and the high accuracy without sacrifice of range.

COMPARISON WITH OTHER METHODS

In comparing the results in the tables with those of other methods, it is important to remember that these† are results on a single unknown, when many unknowns were determined from a single control and a single known; whereas the results reported for most methods are the averages of several replicates or dilutions of an unknown compared with a series of controls and knowns.

*When $(2.000 - \log_{10}T)$ is the formula for deriving optical density from per cent transmission (T), and 12 mm. is the inside diameter of the Klett-Summerson tube. The Klett-Summerson scale is calibrated in divisions 500 × optical density.

†Except results in Table VI which are based on two knowns.

SUMMARY

A quantitative method of penicillin assay is described which is flexible enough to compare favorably with any published method in simplicity, sensitivity, accuracy, range, specificity, and speed with which results are available.

It is based on turbidimetric determination, using a new principle of computation.

It is adapted to all types of penicillin assay now made and to quantitative assay of other potent bacteriostatic or bactericidal agents. Techniques for streptomycin and trivalent arsenicals are given.

The method has the advantage of employing materials and supplies already available in most laboratories and techniques with which the average laboratory technician is familiar. Results of plus or minus 25 per cent accuracy may be available within 2 hours and of plus or minus 10 per cent accuracy within 2.5 to 3 hours. For most applications there is no interference by penicillin-resistant contaminants. It will detect as little as 0.001 unit per cubic centimeter of penicillin with plus or minus 100 per cent accuracy. When performed for maximum accuracy, error can be reduced below plus or minus 5 per cent. It will cover a fourfold range with plus or minus 10 per cent accuracy, a sixfold range with plus or minus 25 per cent accuracy, and a hundredfold range with plus or minus 100 per cent accuracy at the extremes.

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AN EXPERIMENTAL METHOD FOR OBTAINING AN ULTRAFILTRATE OF THE BLOOD

M. R. MALINOW* AND W. KORZON
CHICAGO, ILL.

ULTRAFILTRATION may be a useful approach to the investigation of simultaneous clearances with the kidney and in the study of bound and unbound blood constituents. Ultrafiltration permits extraction of a true ultrafiltrate[1] and can be considered as analogous in principle to glomerular filtration. Geiger[2] has described an ingenious device consisting of two layers of collodion supported by two perforated metal sheets that he connected to the carotid artery of a dog. The blood was circulated between the collodion layers and an ultrafiltrate extracted. It is possible that an improved ultrafilter may have some merit, when properly developed, as a supplement to the excretory function of the kidney.

Our purpose was to develop an ultrafilter which could duplicate glomerular function in a dog, both qualitatively and quantitatively, to be used in an attempt to prolong the life of uremic animals. We decided to use cellophane tubing as the filter membrane, as it appears to have advantages over other materials used in the past. Recent reports have shown that it has a very reproducible and consistent porosity, that sterilization does not greatly alter its permeability, that it has a neutral reaction, and that it can withstand high pressures when properly supported.[3-8] From a practical standpoint cellophane tubing is inexpensive, is available in several thicknesses and diameters, and is relatively easy to handle.

We have constructed an apparatus which permits the flow of blood from an animal through cellophane tubes arranged to provide a maximal filtering surface per unit volume of blood. To increase the rate of water and crystalloid extraction, the cellophane tubes are submitted to an effective filtering pressure of approximately 800 mm. of mercury. This equipment has been successful in collecting the ultrafiltrate of circulating blood in quantities up to 1,200 c.c. per hour.

DESCRIPTION OF THE APPARATUS

The apparatus consists of a filter, a vacuum chamber, and a pump to circulate the blood. These are illustrated in Figs. 1 to 8 and details are given in the legends.

The Filter (Figs. 1 to 4).—The filter is made up of twenty identical filter tubes connected in parallel. Each filter tube consists essentially of a flat cello-

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*H. Gradle Fellow from Buenos Aires, Argentina.

phane tube* passed between two strips of nylon tape† which are sewed together down their full length. This is then sandwiched between two stainless steel strips which are held together by a slotted clamping bar on each side. Filtering pressure is obtained by placing the completely assembled filter tube in a vacuum chamber, bringing the blood inlet and outlet tubes out through a seal, rather than by subjecting the blood to an increased positive pressure.

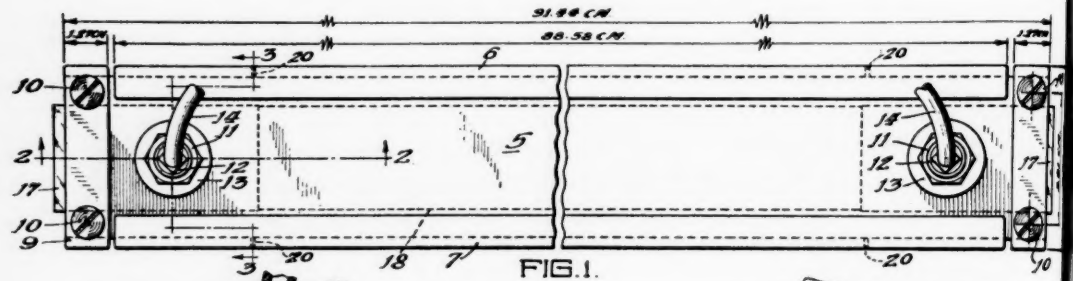

FIG. 1.

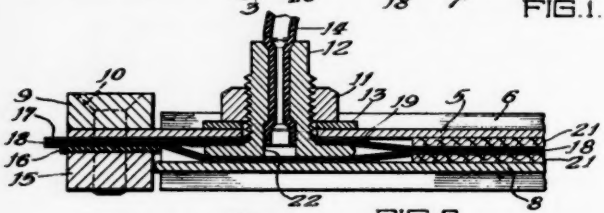

FIG. 2.

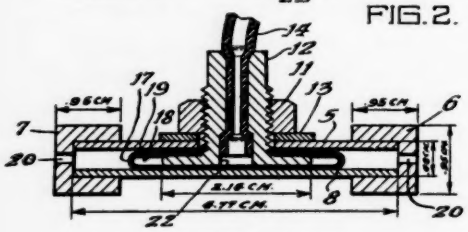

FIG. 3.

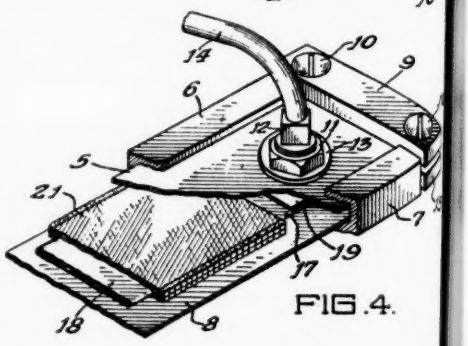

FIG. 4.

Figs. 1 to 4.—Details of the filter: A cellophane tube (*18*) 21.4 mm. in diameter and 0.02 mm. in thickness is passed between two strips of nylon tape (*21*), which are sewed down their full length on both sides so as to form a continuous tube over the cellophane. A short piece of cellophane tubing (*17*) is passed over each end of the previous one. Two flanged stainless steel connections (*12*),* with their rubber tubes (*14*), are passed through holes previously pierced in the cellophane tube. A thin rubber gasket (*19*) is placed over the flange of each connection, and the latter are then screwed to a stainless steel strip (*5*) with a washer (*13*) and a nut (*11*). This strip has two holes in it, 11 mm. in diameter and 83.9 cm. apart, for the purpose. Each end of the cellophane tube is closed with two stainless steel bars (*9* and *15*) with a rubber gasket (*16*) between them. These bars are fastened with two screws (*10*). Another strip (*8*), of the same width as (*5*) but 2.8 cm. shorter, is placed under (*5*) and both are inserted into the slots milled in the clamping bars (*6* and *7*). Holes (*20*), 1 mm. in diameter, at intervals of 9.5 cm. permit the drainage of the filtrate.

*Between the flange of the connection (*12*) and the cellophane tube (*18*) there is a space, not shown in Figs. 2 or 3, through which the blood finds its way when it circulates within the filter.

The principle of the filter is simple. The cellophane tube through which the blood circulates acts as a semipermeable membrane. It is separated from the steel strips by the nylon tape which, by virtue of its weave, forms a porous chamber through which the vacuum can act to reduce the pressure on the exterior of the cellophane tube.

*Manufactured by Visking Co., Chicago, Ill.

†Kindly supplied by Phoenix Trimming Co., Chicago, Ill.

The capacity of each filter tube is 30 c.c., but this can be reduced by placing filter paper strips of appropriate thickness between the nylon tape and the steel strips. The filtration surface of each unit is 400 sq. cm., approximately. Under a filtering pressure of 800 mm. Hg and with a blood flow between 15 and 100 c.c. per minute, about 60 c.c. of ultrafiltrate per hour are obtained. If more ultrafiltrate is desired, several filters like the one described may be used in parallel (Fig. 5) connected by a collector tube. If the filter is to be used without

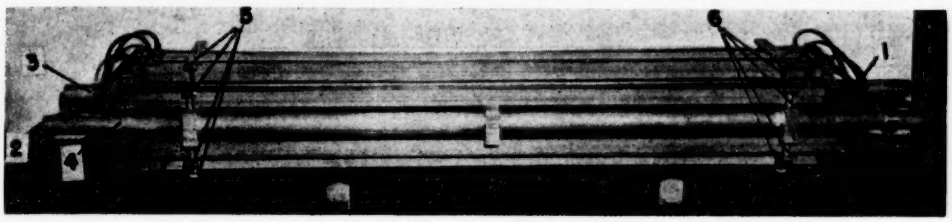

Fig. 5.—Twenty filter units connected in parallel with the collector tubes (*1*), by which blood is distributed to and collected from them. The tube (*2*) serves as an inlet or outlet for the blood in the whole filter. The filter tubes are clamped to an aluminum frame (*3* and *4*) with the metal strips and screws (*5* and *6*).

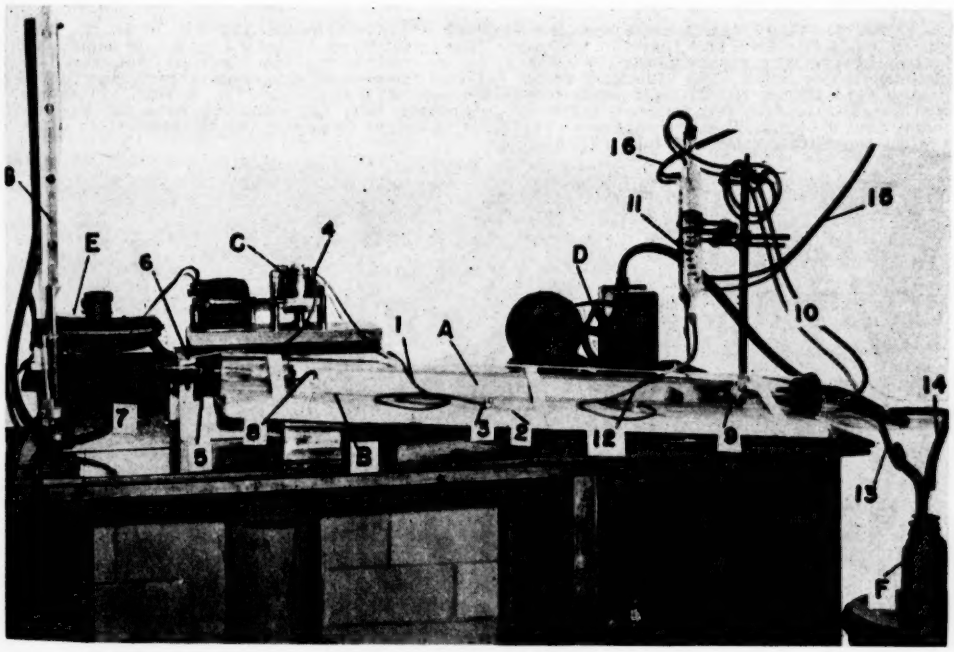

Fig. 6.—The apparatus assembled and ready for use. *A*, A single filter unit; *B*, the vacuum chamber; *C*, the blood pump; *D*, the vacuum pump; *E*, the rheostat; *F*, the bottle for collecting the ultrafiltrate; *G*, a mercury manometer connected to the vacuum chamber. The inlet tube (*1*) is connected through a wide cannula (*2*) and a three-way stopcock (*3*) to the femoral artery of the dog; (*4*) is the special rubber tubing used in the blood pump; (*5*) is the stopper with two tubes; (*6*) is the tube through which blood is pumped into the filter; (*7*) is the tube connected with the mercury manometer; (*8*) is the inlet tube to the filter; (*9*) is the outlet tube from the filter; (*10*) is the outlet tube from the chamber, leading to (*11*) an ordinary condensor used to warm the blood before being returned to the animal through tube (*12*). This latter tube also has a three-way stopcock and a cannula. This cannula is connected with the femoral vein of the animal. The bottle (*F*) receives the ultrafiltrate from the chamber through a rubber tube (*13*) and is also connected to the vacuum chamber by another tube (*14*). Other tubes (*15* and *16*) carry warm water to and from the condenser.

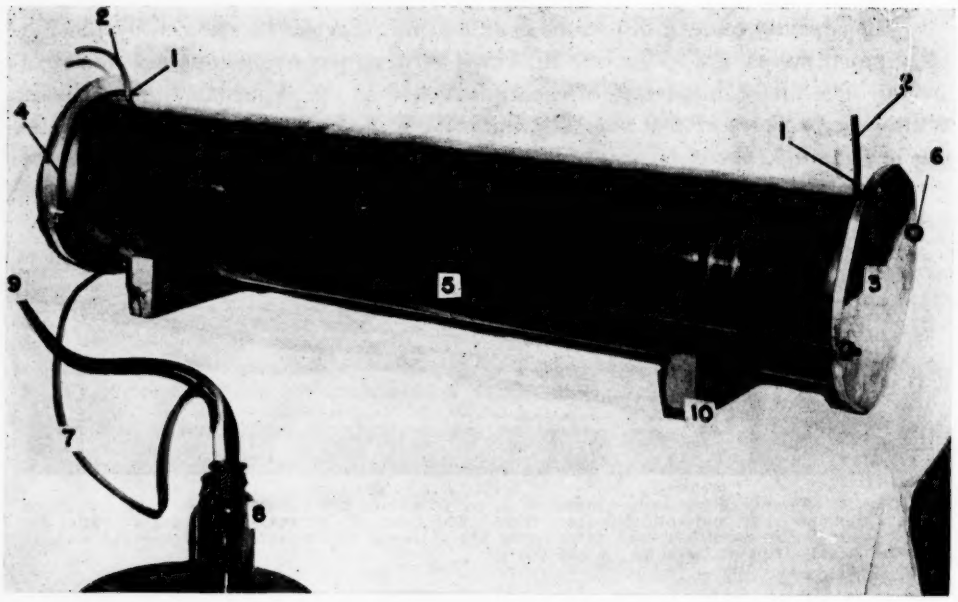

Fig. 7.—The large vacuum chamber employs a brass cylinder, 122 cm. in length, 25.3 cm. in inside diameter, and 3 mm. in thickness. The metal tubes (*1*) of 8.5 mm. bore are slightly smaller than the rubber tubes (*2*) (Fig. 5, *2*), in order to provide a perfect seal when the chamber is evacuated. The aluminum plates (*3*) seal the ends of this chamber with the rubber gasket (*4*) and are tightly held in place with the rods (*5*) and nuts (*6*). A tube (*7*) drains the ultrafiltrate into the collector bottle (*8*). Another tube (*9*) connects with the vacuum pump (not shown.) The two supports (*10*) differ in height to permit the ultrafiltrate to drain toward the filtrate collecting tube (*7*).

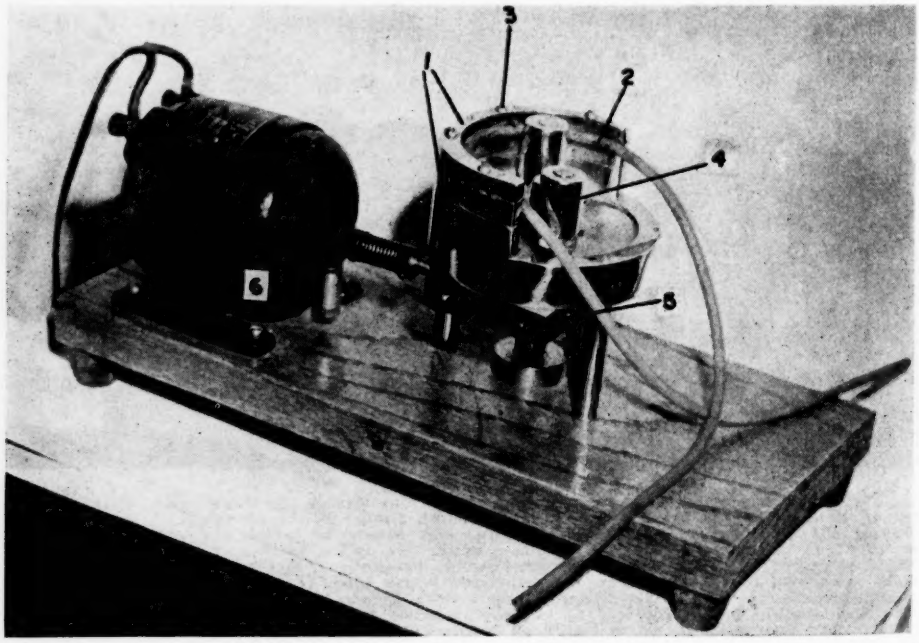

Fig. 8.—The pump consists of two semicircles of plated iron (*1*) between which the special tube (*2*), a rubber tube with a side piece of rubber that is held by the iron semicircles, is fastened with four screws (*3*). Rollers (*4*) milk the tube causing the blood to circulate. Gears (*5*) connect the axis of the roller arm with the motor (*6*).

circulation of blood or when a small amount of blood is to be ultrafiltered, the outlet tube is clamped after the filter has been filled.

The Vacuum Chambers (Figs. 6 and 7).—Two vacuum chambers were used. For use with one or two filter units a long glass tube was employed, which was sealed by a rubber stopper at each end. A glass cannula through each stopper provides a connection for the inlet and outlet tubes of the filter unit, and two more cannulae supply a means of exhausting the tubes and of collecting the ultrafiltrate.

The large chamber, for use with up to twenty filter tubes, is a large diameter brass tube closed at each end by an aluminum disk with a rubber gasket. A seal for the rubber inlet and outlet tubes of the filter is formed by drawing each through a short metal tube which is soldered into the body of the brass tube. The inside diameter of these tubes was made slightly smaller than the outside diameter of the rubber tubing to insure a good seal. Another tube soldered into the brass tube connects with a collector bottle for the ultrafiltrate.

The Blood Pump (Fig. 8).—This pump, similar to the one described by DeBakey,[9] consists essentially of two semicircles of plated iron, one on top of the other, between which a special rubber tube* is clamped. Two rollers, driven by an electric motor through a worm gear, milk approximately 2 c.c. of blood through this tube in each revolution. Among the advantages of this type of pump are that a nonpulsating flow is obtained and that there is little trauma to the blood cells.

TECHNIQUE IN ASSEMBLING THE EQUIPMENT†

The technique in assembling the equipment must be followed rigorously for successful results. A length of cellophane tubing is cut from the roll and is passed between the strips of nylon tape, leaving an extra 15 cm. of cellophane on each side. Two short pieces of cellophane tubing are opened‡ and placed over the ends of the longer tube to re-enforce it at these points. A dusting of starch powder is used to permit the short pieces to slide easily. The long tube is opened and a hole is punched near each end and on the same side of the cellophane tube with a special punch, 11 mm. in diameter, the same distance (83.9 cm.) apart as the holes in the strip (*5*). In order to make these holes, an ordinary wooden tongue depressor is introduced into the long tube and the punch is pressed against it through the double cellophane. The stainless steel connections (*12*) with their rubber tubes are passed through these holes, a thin rubber gasket (*19*) is placed above the cellophane, and the connections (*12*) are screwed tightly to the strip (*5*); then both ends are closed by tightly screwing the two bars (*15* and *9*) together with a rubber gasket (*16*) in between. The strip (*8*) is placed below and both strips are introduced into the slots of the clamping bars (*6* and *7*).

*Obtained from A. S. Aloe Co., St. Louis, Mo.

†The numbers refer to Figs. 1 to 4.

‡Dry cellophane tubing is opened by applying a small strip of adhesive or transparent tape to each side of the flat cellophane tube about 1 cm. from the end, allowing the tape to project a little beyond the tube. The strips of tape are then pulled gently apart, thus separating the cellophane layers at the end of the tube. Blowing into the tube at this point will then open the tube down its entire length.

The unit so assembled is soaked in cool tap water for half an hour and then is checked for leaks by connecting one of the rubber tubes (*14*) with a source of air under approximately 1½ atmospheres of positive pressure. The patency of the filter is checked first by watching the escape of air from the second rubber tube. If the filter is not patent, the air is shut off and turned on again, increasing the pressure slowly. If this does not open the filter, injection of saline with a syringe may accomplish the purpose.

Leaks in the system are manifested by small bubbles coming out while air is injected under pressure with the outlet tube clamped off. These must be differentiated from trapped air in the system. Trapped air disappears after a time but air from a leak continues to escape. There are three sources of leaks:

1. Holes in the cellophane, which were found in one out of ten tubes of cellophane employed by us. Such leaky tubes must be replaced.

2. Inadequate tightening of the connections (*12*) or of the end clamps (*15*). A continuous stream of air bubbles locates the loose connection, and these leaks usually disappear after retightening.

3. Wrinkles in the cellophane at the ends (*17*). Under these conditions air continues to escape despite retightening. In such cases removing the end pieces (*15*) and smoothing out the cellophane generally corrects the leakage.

The connections (*12*), all rubber tubing, glass cannulae, and adaptors used in the experiment, were rendered pyrogen-free by boiling for thirty minutes in 4 per cent NaOH, rinsing with tap water, boiling for fifteen minutes in 0.5 per cent acetic acid, and finally rinsing again with tap water.

CHARACTERISTICS OF THE FILTER TUBE

In order to determine its characteristics, a filter tube was placed in the glass vacuum chamber and the inlet and outlet tubes each were connected with a graduated bottle containing citrated human blood. Altering the relative height of the two bottles changed the pressure and the rate and direction of blood flow. Further pressure and flow changes were obtained by using a screw clamp on the outlet tube.

Filtration under the same conditions was performed with two filter tubes which were identical except that the capacity of one was 15 c.c., the other 30 c.c. The filtration rates of both were the same. Nevertheless, when a large number of units are used in parallel, the total amount of ultrafiltrate is sometimes less than would be obtained if the same number of units were functioning separately. The probable reason for this is that, as the resistance of a large number of filter tubes in parallel is very low, with a relatively small blood flow (up to 150 c.c. per minute for 20 units) some of the tubes are not supplied with circulating blood and so do not function.

No change in the filtration rate was observed: (1) as a result of sterilization* with steam at 250° F. for fifteen minutes; (2) with continued filtration

*Sterilization was performed by connecting the inlet of the filter with a pressure cooker after the desired temperature and pressure was attained and clamping the outlet of the filter.

over ten hours; (3) with blood flow varying from between 15 and 100 c.c. per minute; and (4) with ultrafiltering liquids of different viscosity (namely, blood, and saline solution).

The blood used in filtration was examined microscopically on a number of occasions after centrifuging, and no hemolysis was observed.

Using a vacuum of 35 mm. Hg and varying the positive pressure applied to the circulating blood, the following results were obtained in the same filter:

EFFECTIVE PRESSURE IN MM. Hg	FILTRATION RATE IN C.C. PER HOUR
760	54
760	56
780	60
805	60
825	60
825	59
875	63
925	70

The filtration rate was 5 c.c. per hour in another experiment in which only 75 mm. Hg of positive pressure was applied and no negative pressure was used.

As expected, these observations show that there is a proportionality between the pressure and the amount of ultrafiltrate formed.

NATURE OF THE ULTRAFILTRATE

The ultrafiltrate obtained from human blood and that of dog blood were analyzed in several instances for urea, nonprotein nitrogen, and proteins. It was found to be protein-free and with the N.P.N. and urea in equilibrium with the blood. In later experiments employing dogs certain other ultrafiltrate constituents were analyzed and the results are shown in Table I.*

ACTION OF THE ULTRAFILTER WHEN CONNECTED TO A DOG

Method.—Two types of experiments were performed. In the first type, a single ultrafilter tube was connected with a normal dog. In the second, the large unit with 20 such units in parallel were connected with a nephrectomized, uremic dog. For the latter type of experiment the dogs were bilaterally nephrectomized three days previously; they were injected daily with 100 c.c. of 50 per cent solution of glucose intravenously and with 100 c.c. of 5 per cent glucose, 50 c.c. of saline, and 50,000 units of penicillin subcutaneously. Blood volume determinations were made at the beginning and at the end of the experiment. Blood samples were taken immediately before and once daily after the nephrectomy, at the beginning of the experiment, one hour afterward, and at the end. Samples of the ultrafiltrate were taken simultaneously with the blood collections during the filtration. Chlorides, urea N, creatinine, CO_2 capacity, proteins, glucose, and hematocrit were determined in some of these samples. The

*The methods used were: urea nitrogen: Ormsby, A. A.: J. Biol. Chem. 146: 595, 1942; proteins: Weichselbaum, T. E.: Am. J. Clin. Path. 16: 40, 1946; creatinine: Folin, O., and Wu, H.: J. Biol. Chem. 38: 81, 1919; chlorides: Wilson, D. W., and Ball, E. G.: J. Biol. Chem. 79: 221, 1928; carbon dioxide capacity: Van Slyke, D. D., and Cullen, G. E.: J. Biol. Chem. 30: 289, 1917; blood volume: single samples in duplicate twelve minutes after injection of the dye (T1824); hematocrit: 10,000 r.p.m. during ten minutes in Winthrobe tubes.

These determinations were carried out in the Department of Biochemistry through the kindness of Dr. C. Cohn.

TABLE I. OBSERVATIONS ON MALE DOG, WEIGHT 27 POUNDS, RENDERED ANEPHRIC AND CONNECTED TO ULTRAFILTER*

REMARKS		UREA N (MG./100 C.C.)	CREATININE (MG./100 C.C.)	PROTEIN (MG./100 C.C.)	CO_2 (VOL. %)	HEMATOCRIT (%)	Cl (MG./100 C.C.)
Preoperative	Blood	21		6.9	45	48	630
24 hr. after nephrectomy	Blood	65			32	47	680
48 hr. after nephrectomy	Blood	180		5.5	58	44	600
72 hr. after nephrectomy	Blood	175		5.4		38	540
After 1 hr. of filtration	Blood	135					590
	Plasma	145	8.6			42	590
After 8 hr. of filtration	Blood	75		3.9		39	640
	Plasma	83	6.0				600
Ultrafiltrate obtained at end of 1 hr.		160	7.2				660
Ultrafiltrate obtained at end of 8 hr.		80	5.0				660

	BEFORE FILTRATION	AFTER FILTRATION
Plasma volume	964 c.c.	1010 c.c.
Blood volume	1550 c.c.	1650 c.c.
Hematocrit	38%	39%

Total amount of filtrate, 7,200 c.c. collected in eight hr.
Total amount of urea N "excreted," 6.9 Gm.
Urea clearance (concentration in the blood assumed to be 125 mg./100 c.c., the mean between 175 and 75), 11.3 c.c./blood/minute.

*In this experiment the lag seen in Fig. 9 and explained in the text has not been taken into consideration. This lag explains the lack of parallelism between the plasma and ultrafiltrate concentrations of urea and creatinine.

samples collected after one hour represent the results of the mixing with the donor's blood in the filter and the result of one hour of filtration. Fluid balance during the filtration was maintained by injecting intravenously every ten minutes an amount of Ringer-Krebs solution* equal to the amount of ultrafiltrate collected during the interval.

In both types of experiment the inlet of the filter was connected with the central end of the femoral artery and the outlet with the central end of the femoral vein, the blood being circulated by means of the pump† previously described. The effective filtering pressure used was approximately 800 mm. of mercury. The dogs were injected with 40 to 50 mg. of heparin‡ immediately before the experiment to prevent blood coagulation and were narcotized with morphine, with a 1 per cent solution of procaine used locally.

In order to help avoid the formation of clots the cannulae used were as large as possible. They were washed with concentrated nitric acid before the experiment, cleaned with 4 per cent NaOH, and then rinsed in tap water. The system was filled with saline when only one or two filter tubes were used and with compatible dog's blood when more units were employed, in order to avoid air embolism and the possibility that the dog would lose too much blood into the filter. The blood was warmed to body temperature before it was returned to the animal.

*The Ringer-Krebs solution was prepared by mixing the following substances in 100 c.c. of water; NaCl, 6.923 Gm.; KCl 0.354 Gm.; $CaCl_2$, 0.057 Gm.; KH_2PO_4, 0.170 Gm.; $MgSO_4.H_2O$, 0.2925 Gm.; $NaHCO_3$, 2.10 Gm.; Glucose, 1.0 Gm.; O_2 was bubbled through and the temperature was maintained at approximately 38° C.

†With a single filter tube the pump is not always necessary, as the normal arterial pressure is sufficient for the purpose.

‡Liquaemin liberally supplied to the department by Roche Organon, Inc., Nutley, N. J.

Results With Normal Dogs.—Seven normal dogs were subjected to filtration with a single unit for periods ranging from one to five hours. The flow of blood through the filter was 50 to 70 c.c. per minute. All of the animals survived.

Uniformly, the amount of filtrate collected was 60 c.c. per hour, it was always protein-free, and in no case was hemolysis detected macroscopically. The concentration of urea in the blood and in the ultrafiltrate of one typical experiment is shown graphically in Fig. 9. As can be seen, there is a period of forty minutes during which the filtrate shows little urea concentration. This is due to the dilution of the ultrafiltrate by the saline held in the nylon tape. As this saline is replaced by ultrafiltrate, the urea concentration rises sharply and then levels off. The difference between the concentration of urea in the filtrate and that in the plasma may indicate that urea is not wholly diffusible or that it is bound. This possibility now is being investigated.

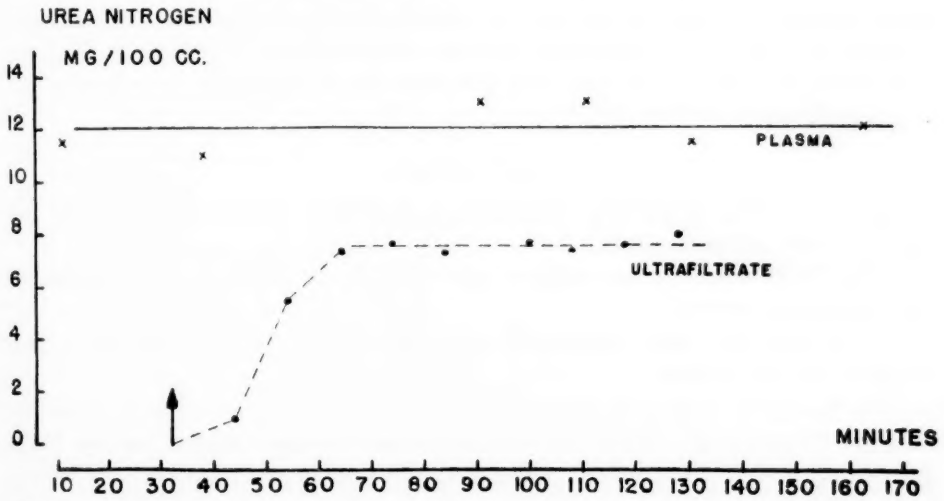

Fig. 9.—A typical experiment performed on a normal dog, showing the urea N concentration in the plasma and in the ultrafiltrate. The arrow indicates the beginning of filtration.

Several deductions can be drawn from these experiments:

1. The filter yields an appreciable amount of ultrafiltrate when connected with a dog.

2. The filter itself is not toxic. The dogs survived the experiment for a long time, and when they were sacrificed, autopsy revealed no macroscopic abnormalities. The limb used was in good condition despite the ligation of the femoral artery and vein.

Results With Uremic Dogs.—Four uremic dogs were used in the second type of experiment. The blood flow through the filter was 110 c.c. per minute with each. Two dogs died accidentally after two and three hours of filtration, respectively; the first died from shock due to blood loss, the second from pulmonary embolism. The other two dogs were filtered successfully for three and eight hours, respectively. They did not appear to be injured by the filtration,

judging from the general appearance and pulse rate, and were up and around when they came out of the anesthesia. These animals died in uremia thirty-six hours after the completion of the experiment. A typical result in one of these dogs is summarized in Table I.

These experiments show that the animals apparently were not adversely affected by the procedure, despite the fact that as much as 7,200 c.c. of fluid was filtered from the blood of a dog (and replaced by Ringer-Krebs solution) with a blood volume of 1,550 cubic centimeters. The chlorides returned to the normal level and the hematocrit remained constant during the experiment. Urea and creatinine were reduced by this ultrafiltration. Nevertheless, the fact that only 7 Gm. of urea were cleared in eight hours makes this procedure with the apparatus employed unsuitable for man, even though its use in dogs may considerably reduce the blood urea. A larger ultrafilter with more filtering surface, or one employing a much greater filtering pressure, may have a sufficient clearance of urea for man, but the dialyzer described by Kolff appears superior in simplicity and in obtainable results because the steeper gradient between urea concentration in the blood and the outside fluid dialyzed into seems to produce a greater urea clearance per unit area of cellophane than the ultrafiltration method described.

CONCLUSIONS

An ultrafilter employing cellophane is described and its applicability for experimental purposes noted.

The filter described has definite advantages over previous ones because of the following reasons:

1. It provides a large amount of ultrafiltrate in comparison with the volume of blood of the animal.

2. The blood in the filter can be placed under a wide range of pressures and can be circulated within the filter at different rates without the red blood cells being unduly traumatized. The filter does not lead to macroscopic hemolysis of the blood.

3. It is easily sterilized.

This ultrafiltration method, however, has limitations in its use for the control of uremia, especially since it has such low clearance values compared with the values reported by Kolff[13, 14] for dialysis.

Such a filter may be useful in the investigation of simultaneous clearances with the kidney and in the study of bound and unbound blood constituents. The composition of ultrafiltrates obtained during this investigation favors this opinion.

We wish to express our gratitude to Dr. L. N. Katz for his advice and guidance and to acknowledge the considerable assistance given us by other members of the department.

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BOOK REVIEWS

Penicillin in Syphilis. By *Joseph Earle Moore,* M.D., Associate Professor of Medicine and Adjunct Professor of Public Health Administration, the Johns Hopkins University; Physician-in-Charge, Syphilis Division of the Medical Clinic, and Visiting Physician, the Johns Hopkins Hospital; Chairman, Syphilis Study Section, National Institute of Health, United States Public Health Service; Chairman, Subcommittee on Venereal Diseases, National Research Council. Charles C. Thomas, Publisher, Springfield, Ill., 1946. Price $7.50. Cloth with 731 pages, 109 illustrations, 122 tables.

The startling discovery of the treponemicidal action of penicillin by Mahoney, Arnold, and Harris in midsummer of 1943 resulted in the almost immediate organization of a nation-wide cooperative study of the efficacy of this antibiotic in syphilis. This collaborative effort, which is perhaps unparalleled in medical history, was motivated by the urgent necessity of the Armed Forces for authoritative information regarding the effectiveness of this agent. Although the facilities of eight laboratories of experimental syphilis were enlisted for intensive study of penicillin in rabbit syphilis, time did not permit final definition of the time-dose relationship in the experimental animal before human trial was undertaken. Accordingly, clinical investigation has proceeded simultaneously in a total of forty-four clinics and treatment centers. Thus far, these participating agencies have treated some 25,000 patients with early syphilis by assigned schedules (thirty-six all told) in a uniform manner, results being analyzed as a group by a central statistical unit.

Since the outset of the study Moore in his capacity as Chairman, first of the Penicillin Panel of the National Research Council and more recently of the Syphilis Study Section of the United States Public Health Service, the sponsoring organizations, has occupied a position uniquely fitting him for authorship of this monograph. As a consequence, he has had access to material restricted during the war years, some of which still is unreported, as well as to unpublished data of the individual cooperating groups. The author thus has been able to assemble under one cover all the information relating to the effect of penicillin in syphilis available as of October, 1946.

As stated in the preface, "The author's only excuse (for this monographic presentation of the admittedly preliminary results now available*) can be that so far, the medical profession has been informed only that penicillin is of value in syphilis. How best to employ it in the several stages of syphilitic infection, on the basis of current knowledge, has nowhere yet been systematically presented. This monograph is an effort to supply the deficiency. It is prepared with full knowledge of its inadequacies and of the necessity for frequent revision to maintain it up to date."

The material is presented in nineteen chapters—actually eighteen, since one (the last) is concerned with streptomycin. Of these, the first seven are devoted to fundamental information—the chemistry of the penicillins, pharmacology, toxicity, therapeutic efficacy, etc. For the student of syphilis the author's exposition of these basic data is unquestionably of greater value than the chapters concerned with clinical use. Of particular interest is the discussion of the results obtained by the cooperating laboratories in experimental syphilis and the implications of these results as to the mode of action of penicillin against *Treponema pallidum.* The experimental data thus far available suggest, and this is not belied by the accumulating clinical results, that the *desideratum* in syphilis may not be large dosages over short periods of time but smaller dosages prolonged for weeks or perhaps even months. If these indications are borne out by further study, the hope of a quick, safe (that is, with penicillin alone) cure for early syphilis suitable for mass application may prove to be unattainable.

*Parenthetic words are the reviewer's.

One chapter is concerned with the administration of penicillin for other diseases as a source of confusion in the diagnosis of syphilis. The suppressing effect of penicillin administered for the treatment of gonococcal infections on the early manifestations of syphilis is emphasized, as is the necessity for prolonged serologic follow-up (at least four months) of penicillin-treated patients with gonorrhea, to rule out simultaneously acquired syphilis.

The remaining ten chapters recount the accumulated information pertaining to the clinical use of penicillin in the various stages of syphilitic infection. Since, from the standpoint of the Army and Navy during the war years, early syphilis and the various types of late neurosyphilis presented the greatest therapeutic problems, emphasis has been directed toward defining the place of penicillin in the treatment of these stages of the disease. The early syphilis results are those accumulated prior to August, 1945, based on the treatment of approximately 11,000 patients by twenty-six treatment regimens. At the time this book was written, patients treated since the date mentioned had been followed too short a period of time for even preliminary evaluation. The results of penicillin treatment of early syphilis may be summarized with the statement that the optimum regimen of treatment with penicillin (sodium or calcium suspended in peanut oil and beeswax), either alone or combined with metallotherapy, still is not defined since the over-all cumulative failure rates of even the best schedules approximate 15 per cent at the end of one year. Suggested schedules utilizing larger and/or longer dosage schedules than those so far analyzed are appended by the author who implies, though it is not so stated, that the results may be no better than with smaller amounts.

Scattered information based on small series of patients indicates that penicillin is effective in early syphilis resistant to arsenic and bismuth, in benign late syphilis, and in at least one type of ocular involvement (the iritis of early syphilis); however, in others such as interstitial keratitis and primary optic atrophy, results are only partially successful. In view of the absence of serious toxic reactions few will disagree that penicillin is the treatment of choice of the pregnant woman with syphilis and probably also of the infant with early congenital syphilis. Little or no information is available as to the efficacy of penicillin in either latent or cardiovascular syphilis since these are both long-term projects. In this respect, however, the physician is well advised that penicillin is no more effective than combined arsenical and bismuth therapy in rendering the blood test negative, since in late syphilis seroresistance is the rule regardless of the agent employed.

The discussion of penicillin in neurosyphilis reflects more than elsewhere the author's own experience, past as well as present, in the treatment of these complex syndromes. The results with penicillin, even though preliminary, demonstrate conclusively that this agent exerts a profound effect on the spinal fluid abnormalities, the improvement in these elements continuing for many months after treatment. At the present time major discussion centers around the question of whether penicillin plus fever offers any advantage over penicillin alone. Comparative data are scanty, and, although the trends of the spinal fluid syndromes presented by the author suggest that combined treatment *may* be slightly superior, the results for the most part are not statistically significant. Until more evidence has been accumulated many syphilotherapists will continue to regard the superiority of combined penicillin and fever as not proved; but few will disagree that for the present the combination should be recommended for the graver parenchymatous forms of neurosyphilis. There undoubtedly will be dissenters to the author's opinion that induced malaria holds any advantage over fever therapy by mechanical means.

Sufficient has been said to indicate that Moore has presented in detail the available information about the effect of penicillin in syphilis. It is to be hoped that this book will serve to correct many misconceptions regarding the use of penicillin in this disease and the results to be expected therefrom. It was inevitable that, in a few respects at least, the book would be outdated already on the day of publication. This fact does not detract from the vast fund of basic data that the book presents, and this reviewer, for one, hopes that the author's promise of frequent revision will be fulfilled.

VIRGIL SCOTT.

Diseases of the Retina. By *Herman Elwyn*, M.D., Senior Assistant Surgeon, New York Eye and Ear Infirmary. The Blakiston Company, Philadelphia, 1946. Price $10.00. Cloth with 587 pages, 170 illustrations, 19 in color.

The author has brought up to date the clinical picture of retinal diseases, correlating the abnormal physiology with the subjective and objective findings of the subject. The course of each disturbance is followed through the life picture of the disease. Treatment is discussed concisely from the viewpoint of the ophthalmologist, who is familiar with the closely associated fields of internal medicine and neurology.

The book is divided into eight parts: I. Diseases of the Retina Resulting From Disturbances in Circulation; II. Diseases of the Retina Resulting From Vascular Malformations; III. Degenerative Diseases of the Retina on a Hereditary Basis; IV. Inflammatory Diseases of the Retina; V. Tumors of the Retina; V. Diseases of the Retina Leading to Retinal Detachment; VII. Developmental Anomalies of the Retina; VIII. Radiation Injuries of the Retina.

The present-day concept of vascular disease per se, along with the primary vascular change occurring in inflammatory, metabolic, and degenerative diseases, has been handled extremely well. The author has followed a definite plan in presenting his material in an orderly and concise manner with the minimum expenditure of words.

This volume belongs in the library of all ophthalmologists, internists, and neurologists.

WILLIAM M. JAMES, M.D.

ANNOUNCEMENT

REVISED BIOLOGICS REGULATIONS

It is desired to call attention to a revision of the regulations governing the manufacture and sale of biologic products published in the Federal Register on Jan. 21, 1947, and effective thirty days after that date.

As is indicated in these regulations, the scope of the Biologics Law is broadened somewhat, and certain diagnostic products administered or applied to a person or prepared from or with the aid of a biologic product now should be distributed in interstate commerce only if prepared by a licensed laboratory.

Also requiring a license for distribution are blood grouping and Rh typing serums, for which minimum requirements have been prepared in anticipation of the revision of the regulations.

Information as to the status of any of these products, or the requirements applicable to them, may be obtained from the Biologics Control Laboratory, National Institute of Health, Bethesda 14, Md.